UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

TUBERCULOSIS INFECTION IN CAPTIVE SLOTH BEARS (*MELURSUS URSINUS*)

- A PILOT STUDY ON DIAGNOSTIC STRATEGIES

MARIA ISABEL RIBEIRO PEREIRA

CONSTITUIÇÃO DO JÚRI

Doutor Fernando Jorge Silvano Boinas

Doutor Virgílio da Silva Almeida

Doutor João Nestor das Chagas e Silva

ORIENTADOR

Attur Shanmugam Arun, B.V.Sc., M.V.Sc.

CO-ORIENTADOR

João Nestor das Chagas e Silva, PhD

2016

LISBOA

UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

TUBERCULOSIS INFECTION IN CAPTIVE SLOTH BEARS (*MELURSUS URSINUS*)

- A PILOT STUDY ON DIAGNOSTIC STRATEGIES

MARIA ISABEL RIBEIRO PEREIRA

DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

CONSTITUIÇÃO DO JÚRI

Doutor Fernando Jorge Silvano Boinas

Doutor Virgílio da Silva Almeida

Doutor João Nestor das Chagas e Silva

ORIENTADOR

Attur Shanmugam Arun, B.V.Sc., M.V.Sc.

CO-ORIENTADOR

João Nestor das Chagas e Silva, PhD

2016

LISBOA

“Be the change you wish to see in the world”

-Mahatma Gandhi

Acknowledgements

To my parents, who have been the most essential foundation through this whole journey, and for never, ever, finding that my dreams could not go beyond that. To my sister Ana Maria, for simply being the best person that I could have ever asked for to have by my side, since I remember existing. This victory belongs to us four.

To Dr. Arun, for everything you taught me, showed me and entrusted me, for all the missions you let me engage in, all the precious research materials and pictures you provided me, and the long distance effort you endured for me to have this work done. I could not have done this without your help and support.

To Professor Nestor for all the amazing support, for always having a kind word to say, for allow me to go along on your field trips, and, overall, for your never-ending enthusiasm with my Indian wild adventures. You were exactly the person I needed with me in this.

To Professor Telmo Nunes, for your availability in helping me with the study analysis chapter of this dissertation, your great advice and the cheerful words.

To Professor Virgílio Almeida, for impersonation in the best way possible what it really means to be a teacher.

To Wildlife SOS for the externship opportunity and the chance to meet and work with such an inspiring team.

To the Bannerghatta Biological Park Forest Department, for giving permission for me to work at BBRC for so long.

To my nana Celeste and my grandpa Joaquim, though they were not able to watch me finish this mark, will always be a part of the person that chose this path, the one that is now finishing it, and the bigger one that I will become.

To my grandma Helena, my grandpa Carlos, and to the rest of my large 'Ribeiro' family, which always gave me the greatest comfort and joy to be part of. A special acknowledgement to my big cousin Diogo, much more of a friend than a colleague, and much more of a brother than a cousin. Veterinary Medicine will remember us.

To my "since ever" friends, Joana, Mirco, Filipa, Marta and Rita, for continuing to collect along with me, more and more years of growth and friendship, and for it to have survived distance, countless finals seasons and too many weeks of "saudade".

To the friends FMV granted me, Patrícia, Bárbara, Esmeralda, Sara, Gomes, João, Maria and the rest of the "E" class, for making the last six years the best ones yet. You are the true meaning of "second family".

To VETuna, for keeping something so significant as music present in my live, and, overall, for putting in my way people that, otherwise, might have never crossed it. Gonçalo, Joana, Sandra, Madeira, Menino, Pecho, Filipe, Inês and Raquel: we have come a long way together and it was the biggest pleasure to perform by your side.

To the 2014 FAUNA board, for showing me the true meaning of effort, team work and unmeasured dedication for a common goal.

To all BBRC staff, from the keepers to the cook, for everything I experienced with you all. A special thank you to Sridhar and Imam for making me feel like part of the family and for having my back through our journeys across India.

To Susan, my everyday vet partner, for everything you taught me, for all the help, friendship, culinary experiences, and company.

To Catherine, my favorite vet nurse and dear friend, for everything we lived in India, for being an amazing teacher, for your patience, and for showing me how much someone can be passionate about animal's welfare. Sincerest thanks for the grammar revision of this dissertation.

To the BBP Zoo Doctors, Nirupama and Vaseem, for always being so available, professional, friendly, kind and for giving me the precious opportunity of learning and engage in so many procedures at the Zoo.

To everyone at Hospital Veterinário Arco do Cego, Sheila, Joana, Rita, Marta, Mariana, Ivo, Teresa, Mari, Liliana and Catarina, for playing such a huge role in the formation and consolidation of the future 'vet me', and for making the times I spent (and keep spending) in your company, happy ones. A special thanks to Susana, for being the best and most loyal partner in my 2016 veterinary adventure.

To Dr. Lapão from Lisbon Zoo, for allowing me to use and consult the Zoo's Wildlife Medicine precious books. They were very useful.

To Mr. Nuno Silva from Pc Medics, for saving my thesis materials when an informatics tragedy fell upon my pen drive.

Finally, to whoever might read this dissertation in the future with a passionate heart about wildlife and conservation medicine. Hopefully, you will not be disappointed.

Abstract

Tuberculosis infection in captive sloth bears (*Melursus ursinus*)
- A pilot study on diagnostic strategies

Tuberculosis (TB) is a cause of significant morbidity and mortality in both domestic and wild animals, and in humans, remaining a major global public health issue, especially in developing countries as India, the one with the highest TB burden in the world.

Infection by *Mycobacterium tuberculosis* in rescued sloth bears represents a typical case of spillover infection resulting from a prolonged and close cohabitation with infected humans, after being forcibly and illegally poached from the wild as cubs and trained to behave as entertainers in the streets of India. The stress, traumas and hardship that the animals endure, also play a role in the development of the disease, as their immune system is usually compromised.

As in the case of many other wild species, there is critical lack of accredited tests for TB screening in sloth bears. This way, it is of major importance to identify the diagnostic assays that have the highest sensitivity and specificity, in order to achieve a reliable diagnosis and implement a standard methodology.

Various diagnostic methods were used to examine 15 presumable positive animals at Bannerghatta Bear Rescue Centre, in the Karnataka state, India, and their sensitivity was calculated, based on the results. *M. tuberculosis* infection was strongly suspected *ante-mortem*, based on the animals' background as "dancing bears" and the revelation of several positive diagnostic results during their lives. Fourteen out of these 15 bears died between 2008 and 2013 and their death certificate reported *post-mortem* tuberculosis confirmation. Considering the previous statement, it was not possible to calculate the specificity and, thus, obtain a global and underlying insight about the tests in question.

Post-mortem methods present, in general, the highest sensitivity results. According to this study, the *ante-mortem* methods with the most promising results were the ones belonging to the indirect assay category, which are based upon the animal's immune response (both cellular and humoral) instead of the organism detection (as in culture, PCR and microscopy).

A sensitivity increment was achieved when two or three tests from the three major test categories (direct, indirect based on cellular immunity and indirect based on humoral immunity) were used in parallel testing. The highest sensitivity achieved by multiple testing (93.3%) was the same for both double and triple parallel combinations, showing, in this case, no advantages in using combinations of three tests, instead of two, in terms of sensitivity increment.

Key-words: sloth bear, *Mycobacterium tuberculosis*, epidemiology, diagnosis, wildlife, zoonosis.

Resumo

Infeção por tuberculose em ursos-beiçudos (*Melursus ursinus*) em cativeiro - Um estudo piloto em estratégias diagnósticas

A Tuberculose (TB) provoca significativos índices de morbilidade e mortalidade em animais domésticos e selvagens, e em seres humanos, representando um considerável problema de saúde pública, sobretudo em países em vias de desenvolvimento, como a Índia, neste momento aquele com a incidência mais elevada de tuberculose em todo o mundo.

A infeção pelo *Mycobacterium tuberculosis* em ursos-beiçudos em cativeiro, traduz a típica ocorrência de infeção acidental, resultando da prolongada e próxima co-existência com seres humanos infectados, após serem forçada e ilegalmente capturados do seu habitat natural, enquanto crias, e treinados para se comportarem como animadores nas ruas da Índia. O stress, traumas e adversidades passados por estes animais, são fatores no desenvolvimento desta doença, uma vez que o seu sistema imunitário se encontra normalmente comprometido.

Como em muitas outras espécies selvagens, existe uma enorme lacuna no que diz respeito a métodos de testagem de tuberculose em ursos-beiçudos. Deste modo, é da maior importância definir os testes que possuem a sensibilidade e especificidade mais elevadas, potenciando a obtenção de um diagnóstico fiável e de uma metodologia padronizada.

Diversos métodos diagnósticos foram aplicados em 15 animais presumivelmente positivos e residentes no Bannerghatta Bear Rescue Centre, em Karnataka, na Índia, e a sua sensibilidade foi calculada, com base nos resultados obtidos. A infeção por *M. tuberculosis* era fortemente suspeitada *ante-mortem*, com base na proveniência e passado dos animais como “dancing bears” e em vários resultados diagnósticos positivos durante a sua vida. Catorze destes 15 ursos morreram, entre 2008 e 2013, e o seu relatório de óbito reportou a confirmação *post-mortem* de tuberculose. Desta forma, não foi possível calcular a sua especificidade e, assim, obter um conhecimento global e aprofundado dos testes em questão.

Os testes *post-mortem* apresentam, em geral, as sensibilidades mais elevadas. Segundo este estudo, os testes *ante-mortem* com os resultados mais promissores pertencem à categoria de métodos indiretos, baseados na deteção da resposta imunitária do próprio animal (tanto celular como humoral), ao invés da deteção do organismo (como em cultura, PCR e microscopia).

Um aumento da sensibilidade foi conseguido quando dois ou três testes das três principais categorias exploratórias específicas (diretos, indiretos baseados em imunidade celular e indiretos baseados em imunidade humoral) foram usados em testagem paralela. A sensibilidade mais elevada obtida por uma combinação de testes (93.3%) foi a mesma para combinações paralelas duplas e tripla, demonstrando, neste caso, que não existem vantagens em combinar três testes, em vez de dois, no que toca ao aumento de sensibilidade.

Palavras-chave: urso-beiçudo, *Mycobacterium tuberculosis*, epidemiologia, diagnóstico, vida-selvagem, zoonose.

Table of Contents

List of Figures

List of Tables

List of Graphics

List of Abbreviations and Symbols

ºC – Degrees Celsius

µg - Microgram

AAZV - American Association of Zoo Veterinarians

ABRF - Agra Bear Rescue Facility

AFB - Acid-fast bacillus

am- Ante-meridian

AMTD - Amplified *Mycobacterium tuberculosis* direct test

BAL - Broncho-Alveolar Lavage

BBRC - Bannerghatta Bear Rescue Centre

BBP - Bannerghatta Biological Park

BCG - Bacillus Calmette-Guérin

CBC - Complete Blood Count

CI - Confidence Interval

CITES - Convention on International Trade in Endangered Species of Wild Fauna and Flora

CMI - Cellular-mediated immunity/immunologic

CRP - C-reactive protein

CT - Computed tomography

DCs - Dendritic cells

DNA - Deoxyribonucleic acid

DPP- Dual-path platform assay

EAZWV - European Association of Zoo and Wildlife Veterinarians

ELIZA - Enzyme-linked immunosorbent assay

EMB - Ethambutol

EN - Endangered

FVM - UL – Faculty of Veterinary Medicine – University of Lisbon

HE- Hematoxylin and eosin

HIV - Human immunodeficiency virus

HPA - Hypothalamic-pituitary-adrenal

IFN-γ - Interferon-gamma

IGRAS -Interferon-gamma release assays

IM- Intra-Muscular

INH - Isoniazid

IUCN- International Union for Conservation of Nature

LC - Least Concern

LJ - Löwenstein-Jensen

LT- Lymphocyte transformation

LTT - Lymphocyte transformation test

MAPIA- Multi-antigen print immunoassay

MDR - Multi-drug resistance

mg/Kg- Milligram per kilogram

MGITs- Mycobacterial growth indicator tube

mL- Milliliter

MRI - Magnetic resonance imagery

PCR- Polymerase Chain Reaction

PET - Positron emission tomography

pm – Post-meridian

PZA- Pyrazinamide

RFLP – Restriction fragment length polymorphism

RIF- Rifampin/ Rifampicin

RNA - Ribonucleic acid

RNAr- Ribosomal ribonucleic acid

sbsp. - Subspecies

SC - Subcutaneous

SM - Streptomycin

SNS - Sympathetic nervous system

spp. - Species

TB- Tuberculosis

Th- T Helper Lymphocytes

TNF- Tumor Necrosis Factor

TST- Tuberculin skin test

USAHA- United States Animals Health Association

VU - Vulnerable

WHO - World Health Organization

WSOS - Wildlife SOS

ZN - Ziehl-Neelsen

Chapter I- Introduction

Conservation medicine enables us to rethink the ties between human, animal and environmental health (Verma-Kumar, Abraham, Dendukuri, Cheeran, Sukumar & Balaji, 2012). The recognition of this crucial interdependence has led to the multi-disciplinary concept of “One World, One Health, One Medicine”. Pathogens that are transmitted between wildlife, livestock and humans represent major challenges for human and animal health, as well as for wildlife conservation (Maas, Michel & Rutten, 2013).

Tuberculosis (TB) is a cause of significant morbidity and mortality in both domestic and wild animals worldwide, having a wide host range, which includes non-human primates, elephants and other exotic ungulates, carnivores, marsupials, marine mammals, rodents, amphibians, and birds (Montali, Mikota & Cheng, 2001; Miller, 2008; Fefar et al., 2012). It is also considered an important emerging disease in humans, remaining a major global public health issue (Nolte & Metchock, 1995; Ernst & Wolf, 2006; Olsen, Barletta & Thoen, 2010), with one-third of the human population currently infected (World Health Organization [WHO], 2014) and being the leading cause of death in adults due to a single infectious agent (Alexander, Pleydell, Williams, Lane, Nyange & Michel, 2002; Pfyffer, 2007; Lécu & Ball, 2011; WHO, 2016). Of those deaths, about 95% occurred in countries where resources are more limited, with a majority of cases appearing in India and China (WHO, 2014; Fogel, 2015). The best estimate is that, in 2015, there were 1.4 million TB deaths and an additional 0.4 million deaths resulting from TB disease among HIV-positive people. Also, there were 10.4 million new TB cases (including 1.2 million among HIV-positive people) (WHO, 2016).

Serious and vigorous action plans are presently applied to fight the disease in humans in developing countries, but these are still the places facing notable incidences of TB within the animal populations (Rishikesava, Arun, Chandranaik, Basavarajappa, Giridhar & Renukaprasad, 2008; Lécu & Ball, 2011). Wildlife populations that suffer from tuberculosis infection, most often live in close contact with humans or domestic animals, with the highest prevalence rates occurring in captive animals in zoological collections and the lowest rates in animals that rarely come in contact with human populations (Michel, Venter, Espie & Coetzee, 2003).

Awareness of the importance of tuberculosis in wildlife has been increasing, not only for being a potential reservoir of infection for domestic animals, but also a threat to the health and integrity of valuable and rare wildlife species (de Lisle, Bengis, Schmitt & O’Brien, 2002; Isaza, 2003; Michel et al., 2003). The zoonotic potential of these organisms presents an additional concern for animal handlers, like veterinarians and keepers, and, of course, for public health. Therefore, rapid, accurate diagnosis in wildlife species is imperative (Miller, 2008; Arun et al., 2014).

Although a wide variety of mycobacteria are pathogenic, “tuberculosis” refers to infection with specific organisms that belong to the *Mycobacterium tuberculosis* complex (Backues, 2008; Miller, 2008; Lécu & Ball, 2014). The majority of the mycobacteria from the “tuberculosis complex”

have the ability to infect wild animals whereas the susceptibility, pathogeny and immune responses towards mycobacterial infection vary widely between mycobacteria and host-animal species (de Lisle et al., 2002). The predictability of infection outcome is still hard to presume and, therefore, the eradication of TB is potentially linked to the capacity of early diagnosis in domestic and wild host species (Miller, 2008; Lécu & Ball, 2011).

In India, illegally held sloth bears that were confiscated by wildlife authorities and then moved to rehabilitation centres have been reported to have died from tuberculosis infection (Arun et al., 2014; Kemmerer, 2015). A presumptive diagnosis of mycobacterial infection was initially made based on gross findings. Histopathology and microbiology of caseated nodules within the lungs confirmed a diagnosis of *Mycobacterium tuberculosis* infection (Mehrotra, Bhargava, Choudhary & Mathur, 1999; Arun et al., 2014; Kemmerer, 2015; A.S. Arun, personal communication, April 20th, 2016). Symptoms like progressive weight loss, anorexia, gastritis, enteritis, nasal discharge, cough and weakness were observed prior to death in some of these bears (Renukaprasad et al., 2013; Collins, 2014). Zoonotic transmission from human handlers was speculated to be the origin of the infection (Renukaprasad et al., 2013; Arun et al., 2014; Kemmerer, 2015).

1. Sloth Bears

Bears are mammals belonging to the order Carnivora of the Ursidae family and are geographically widespread within North and South Americas, Europe, and Asia. Within Ursidae, eight species of bears exist in total (Table 1), divided by three different subfamilies: Ursinae[1], Tremarctinae[2], and Ailuropodinae[3] (Yoganadn, Rice & Johnsinhg, 2013; Collins, 2014).

Table 1- Biologic Information of Bears, Order Carnivora, Family Ursidae (Adapted from Collins, 2014) and their classification according to the IUCN Red List of Threatened Species (http://www.iucnredlist.org).

Scientific Name	Common Name	Geographic Distribution
[1]***Ursus americanus*** **(LC)**	American black bear	North America
[1]***Ursus thibetanus*** **(VU)**	Asiatic black bear	Central, Eastern, and Southeastern Asia
[1]***Ursus arctos*** **(LC)**	Brown or grizzly bear	Europe, Asia, and North America
[1]***Ursus maritimus*** **(VU)**	Polar bear	Artic regions of Eastern Asia and North America
[1]***Helarctos malayanus*** **(VU)**	Sun bear	Southeast Asia
[1]***Melursus ursinus*** **(VU)**	**Sloth bear**	**Southeast Asia**
[2]***Tremarctos ornatus*** **(VU)**	Spectacle bear	Andes mountains of South America
[3]***Aiuropoda melanoleuca*** **(EN)**	Giant panda	Central China

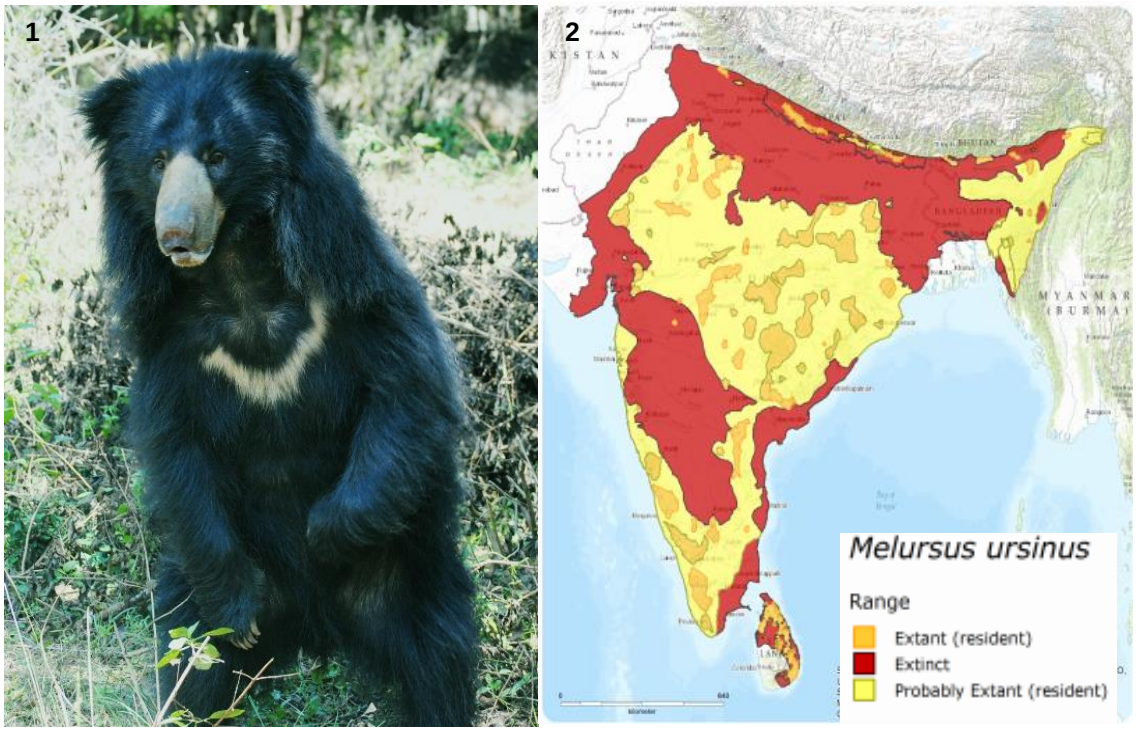
LC – Least Concern; VU – Vulnerable; EN – Endangered[1].

[1] The IUCN divides species into 7 different categories regarding their conservation status: Least Concern (LC), Near Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CE), Extint in the Wild (EW) and Extint (EX). The 2012 "IUCN Red List Categories and Criteria" is available for consult at http://www.iucnredlist.org/technical-documents/categories-and-criteria.

Sloth Bears (*Melursus ursinus*) are a small, usually black with a long shaggy coat, bear species that range across India, Nepal, Sri Lanka, and Bhutan (Figure 2) (Arun, Kajal, Selvaraj & Selvaraj, 2008; Garshelis, Ratnayeke & Chauhan, 2008). In these regions, Pocock (1933) distinguished two distinct races: *Melursus ursinus ursinus* (Figure 1), occurring in continental India and the shorthaired and relatively smaller *Melursus ursinus inornatus*, found only in Sri Lanka. Adult males weigh between 79 and 140 kg, while female sloth bears weigh between 54 and 109 kg (Garshelis, et al., 2008). Bears usually have a longevity of 20-30 years in captivity (von Hohendorff & Giacomini, 2014).

Figure 1- Indian sloth bear (*Melursus ursinus ursinus*) at Bannerghatta Biological Park (Original);

Figure 2- Sloth bear distribution across India, Sri Lanka, Nepal and Bhutan (Source: The IUCN Red List of Threatened Species 2008, http://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T13143A3413440.en).



Sloth bears are reported to exist in 174 Protected Areas in India, which include 46 National Parks and 128 Wildlife Sanctuaries (Chauhan, 2006). Although no truly reliable large-scale population estimates exist for sloth bears, best guesstimates indicate a reasonable possibility of there being ~20.000 or fewer animals, and thus <10.000 adult animals (Garshelis et al., 2008).

These animals subsist primarily on termites, ants, and fruits and are the only bears with specific adaptations for myrmecophagy (ant and termite-eating) (Arun et al., 2008; Garshelis et al., 2008; Yoganand et al., 2013). In captivity, sloth bears are typically fed omnivorous diets, similar to the ones consumed by other bear species (Collins, 2014). Sloth bears are almost nocturnal in their habits due to the human geographic pressure inside protected forest areas and sanctuaries (Seshamani & Satyanarayan, 1997).

Sloth bears are listed as “Vulnerable” by the International Union for Conservation of Nature (IUCN), listed under Appendix I of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), meaning that all trade and exportations are banned and protected under Schedule I of the Indian Wildlife (Protection) Act of 1972 (Arun et al., 2008; Garshelis et al., 2008; Arun et al., 2014). Existing sloth bear populations face direct threats from habitat loss, poaching and capture of cubs (Arun et al., 2008; Garshelis et al., 2008; D'Cruze et al., 2011; Yoganand et al., 2013).

Poaching occurs mainly for the commercial trade of bear parts in the black market, particularly gall bladders, male reproductive organs, bones, teeth, claws and even fat (Arun et al., 2008; Garshelis, et al., 2008; Renukaprasad et al., 2013). Capture of live cubs for use as "dancing bears" by the Kalandar community, represented a major and significant threat in the past (Seshamani & Satyanarayan 1997; Arun et al., 2008; Renukaprasad et al., 2013; Kemmerer, 2015). Evidence suggests that sloth bear populations have declined by 30–49% over the past 30 years as a result of all these threats (Garshelis et al., 2008; D'Cruze et al., 2011).

2. Kalandars and “Dancing bears”

The Kalandars are a widely dispersed, endogamous ethnic group of nomadic entertainers, originally composed by Muslim gypsies, found throughout South Asia (Seshamani & Satyanarayan, 1997; D'Cruze et al., 2011). They make their living using a wide number of performing animals like monkeys, bears, fighting roosters and pigeons and keeping others as pets, such as civet cats, owls, falcons, and partridges to display to their audience (Seshamani & Satyanarayan, 1997).

For over four hundred years, “dancing bears” were a very common sight in India (Figure 3, 4 & 5), being part of an old tradition, where captured bear cubs were initially trained and forced to perform for emperors (Seshamani & Satyanarayan, 1997; D'Cruze et al., 2011; Renukaprasad et al., 2013). For this purpose, cubs were poached from the wild and their mothers killed, most of the times, since they instinctively try to protect their young (Seshamani & Satyanarayan, 1997; Renukaprasad et al., 2013; Kemmerer, 2015).

Figures 3, 4 & 5- “Dancing bears” of India and the Kalandar community (Courtesy of Wildlife SOS, India).

Many times, the bear cubs reached the villages traumatized and dehydrated and, after a couple of weeks, were tied by a rope, to a bamboo pole. The cubs' muzzles were pierced with a large iron needle and a coarse rope was passed through the wound, giving Kalandars the power to easily manipulate the animals, since the wound would never properly heal (Seshamani & Satyanarayan, 1997; Renukaprasad et al., 2013; Kemmerer, 2015).

Cubs' permanent dentition appears when they are around 8-10 months old, which made them more difficult to handle by the Kalandars. For this reason, the canines were removed and young males castrated without the use of anesthesia in either of the procedures (Renukaprasad et al., 2013; Seshamani & Satyanarayan, 1997). The mortality rates were very high at this point (Seshamani & Satyanarayan, 1997).

The major animal suffering and abuse does not lie particularly in the actual "dancing" routine, but in all the other aspects involving the trade: the capturing, transporting and training processes; stressors as heat, dust, and noise; and the urban environment in general, very different from their natural one (Seshamani & Satyanarayan, 1997).

Cubs' feeding routines vary through India, with animals being fed with milk and wheat porridge in North India and Ragi porridge and milk in the South. When the cubs are around six months old, they start being fed with Roti bread[2] mixed in milk in North India and steamed Ragi balls[3] and milk in the South (Seshamani & Satyanarayan, 1997).

This practice was made illegal in 1972 in India but continued for, at least, another 40 years (Seshamani & Satyanarayan, 1997; Kemmerer, 2015).

3. Rescue Centres

Since the late 1990s, national and international wildlife institutions have focused their attention on this issue in India. Their efforts to increase law enforcement, provide lifetime care for confiscated bears, increase public awareness about the issue and provide sustainable alternative livelihoods for bear-owning Kalandars, have resulted in a significant positive effect on the conservation and welfare of sloth bears, as the number of "dancing bears" known to be performing throughout India seems to have declined dramatically over the last two decades, from approximately 1,000–1,200 animals in 1996 to approximately 28 animals in 2010 (Table 2) (D'Cruze et al., 2011; Renukaprasad et al., 2013). Today, there is no record of active "dancing bears" in the streets of India, as stated by Wildlife SOS (A.S. Arun, personal communication, April 20th, 2016).

To this day, hundreds of performing bears have been rescued and given a permanent home and lifetime care in Wildlife SOS rehabilitation centres throughout India (Seshamani & Satyanarayan, 1997).

[2] Flat bread originating from the Indian subcontinent, made from stoneground wholemeal flour, traditionally known as atta, and water.
[3] Wholesome meal in the state of Karnataka and the Rayalaseema region in Andhra Pradesh made with ragi flour and water.

Almost every rescued “dancing (sloth) bear” suffers from an enormous variety of disorders. Deficiency of maternal immunity, physical and psychological stress, poor nutrition, poor living conditions, impaired circadian rhythms and lack of veterinary care created a host of predictable medical problems such as external and internal parasites, rabies, leptospirosis, canine adenovirus, tetanus, degenerative joint disease, maggot wounds, severe gum disease and ocular and dental diseases, namely rotting tooth stumps where canines had been purposefully and brutally broken/removed (Arun, 2013; Kemmerer, 2015; A.S. Arun, personal communication, April 20th, 2016).

Their immune systems are frequently compromised and it soon became notorious that a significant number of animals were found to have severe gross lesions of tuberculosis at *post-mortem* examination. It is presumed that the acquaintanceship between Kalandars and bears, similar to the one observed between elephants and mahouts[4] (Maslown & Mikota, 2015), may have resulted in the animals’ infection, making them spillover hosts (Arun, 2013; Arun et al., 2014; Kemmerer, 2015).

Table 2- Status of “dancing (sloth) bears” in India (1996–2010) (D'Cruze et al., 2011).

State or union territory	**Number of dancing bears recorder (year)**			
	1996	**2001**	**2005**	**2010**
Andhra Pradesh	50	11	72	2
Bihar	86	31	27	5
Chhattisgarh	30	27	21	0
Delhi	36	36	36	0
Haryana	116	8	5	0
Jharkhand	20	20	11	3
Karnataka	88	96	29	2
Madhya Pradesh	29	23	8	1
Maharashtra	36	36	1	2
Rajasthan	126	90	10	0
Uttar Pradesh	430	258	89	13
West Bengal	51	32	37	0
Total	**1098**	**668**	**346**	**28**

The year 2005 if often heralded as the date that TB “emerged” as a disease of concern for sloth bears, after several deaths and *post-mortem* diagnosis in bear rescue centres (A.S. Arun, personal communication, April 20th, 2016).

[4] Elephant rider, trainer, or keeper. A mahout starts as a boy in the “family profession” when he is assigned an elephant early in its life.

4. Content Notice

Considering the lack of published data regarding tuberculosis in sloth bears, this dissertation references TB in humans, elephants, non-human primates and dogs, as these hosts most closely provide models to improve the understanding of the disease as it is observed in sloth bears. For instance, elephants and sloth bears are both treated for TB the same way humans are, which is uncommon in animal species (United States Animals Health Association [USAHA], 2012); preventive measures are very similar between elephants and sloth bears (European Association of Zoo and Wildlife Veterinarians [EAZWV], Tuberculosis Working Group, 2010; A.S. Arun, personal communication, April 20th, 2016); and dogs display many similarities in disease progression, lesions and clinical manifestations (Martinho et al., 2013), possibly for being phylogenetically related to bears.

This dissertation will focus on infection by *Mycobacterium tuberculosis* as it is the predominant disease-causing agent in sloth bears (Arun et al., 2014; A.S. Arun, personal communication, April 20th, 2016), with suitable mentions to *M. bovis* cases mentioned in literature (Collins, 2014).

Chapter II- Training Period Activities

The author's 6th year externship of the Integrated Masters in Veterinary Medicine took place at the Bannerghatta Bear Rescue Centre (BBRC) in the Karnataka state, India. This externship started on September 14th, 2015 and was completed by December 13th, 2015, having the length of 13 weeks.

This training had the supervision of Dr. A. Sha. Arun from Wildlife SOS, BBRC and the co-supervision of Dr. João Nestor das Chagas e Silva, from FMV-ULisboa.

The Bannerghatta Bear Rescue Centre was established in 2005 and is one of two major sloth bear sanctuaries in India, the other one being the Agra Bear Rescue Facility, both managed by Wildlife SOS. It is placed inside the Bannerghatta Biological Park (BBP), with many other free-ranging animals occupying the surrounding areas. The Bannerghatta Biological Park area, besides the Bear Rescue Centre, and the main safari area, also contains a Butterfly Park, a feline Rescue Facility and a Zoological Garden within its premises, with a veterinary team that frequently works along with BBRC veterinarians in both places.

Wildlife SOS was established in 1995 and works actively to protect Indian wildlife, mostly through the rehabilitation of rescued wildlife, rehabilitation of the Kalandar community by providing alternative livelihoods[5], assisting State Forest Departments to prevent poaching from the wild and the rescue of conflict animals[6]. Although Wildlife SOS is mainly known for its work with the "dancing bears" of India, it also has active projects to help leopards, elephants, reptiles and other animals.

BBRC houses 86 sloth bears (Figures 6 & 7) at the present moment that are distributed among five enclosures.

At BBRC, the author followed the routine activities performed by the veterinary staff that included feeding inspections, updating records, deworming interventions and parasitological evaluations, laboratory work, literature research for eventual projects and Master's dissertation purposes, environmental enrichment work, animal conditioning, blowpipe training, animal behavior analysis and surveillance, other routine veterinary treatments, wound dressings and clinical interventions/examinations, using anesthesia, some of which happened at BBP Zoo, and different kinds of surgical procedures.

[5] As an example, Wildlife SOS animal keepers are mostly previous "dancing bear" owners.

[6] In areas where deforestation, usually caused by human population increment, reduced animals' natural habitat, there is a higher change for animals and humans to cross paths, creating the so called "human-animal conflict". For this reason, and despite sloth bears being extremely calm animals, are the bear species responsible for the highest number of human deaths, normally after direct human attacks or threats (von Hohendorff & Giacomini, 2014).

Figures 6 & 7- Rescued sloth bears at BBRC safari at Bannerghatta Biological Park (Original).

The amount of hours spent in each activity is stated in Table 3.

Table 3- Hours spent in each activity during the training period.

Activity	Amount of hours spent
1. Routine Rounds & Prophylactic Measures	217 hours
2. Medical Interventions	97 hours
3. Laboratory Work	52 hours
4. Daily Medical Treatments	85 hours
5. Training/Conditioning	57 hours
6. Environmental Enrichment	23 hours
7. Necropsies	12 hours
8. Rescue Missions	81 hours
9. Individual WSOS Assignments	85 hours
10. Oral Presentations	21 hours
11. Research Work	49 hours
12. Blowpipe Training	15 hours
13. Instruction Day	9 hours
Total	803 hours

Regarding the activities performed, the author had the opportunity to participate in the following:

1. Routine Rounds and Prophylactic Measures: The bears at BBRC are fed twice a day (around 11am and 4pm) with porridge (i.e. semisolid food that consists of a mixture of powdered ragi, jowar, soy bean along with honey, vegetables, milk, eggs and salt). The veterinary staff is responsible for the feedings inspection in every enclosure, to register which animals consumed the porridge or not, and perform the hygiene and cleanness inspection of the enclosures. As the symptomatic phase of TB begins with simple gastritis and skipping food, this observation and registry is highly important. Also, the bears are in free ranging areas during day time which makes the individual animal observation highly difficult. Hence, any abnormality or behavioral changes are best noticed during feeding time. The porridge is prepared in the kitchen which is also a target of daily inspection, as well as the fruits that are fed to the animals. Other activities performed included deworming of the animals, administration of medication and vitamins, weighing the bears, preparation of the Operation Theater (OT) before every medical procedure and its cleanup afterwards, and taking part in the daily fruit feeding safari, where enrichment fruit (mostly watermelon) is given to all the bears that have access to the BBP safari area.

2. Medical Interventions: The preparation of all the materials needed for the anesthetic intervention, like the darts with the right amount of xylazine (2 mg/kg) and ketamine (5 mg/kg), according to the animal's last registered weight, is one of the veterinarians' responsibilities. Shortly after the animal is sedated, with the use of the blowpipe (IM route), the animal is moved from its enclosure to the operation theater, where the veterinarians open an IV route, providing fluids to the animal, collect blood from the jugular, and perform a general health examination, otoscopic and ophthalmologic examination, routine ultrasound (US) examination (Figure 8), dental X-ray, if required and collection of other body samples (hair, urine, tracheal/vaginal/rectal swabs) for further analysis either within BBRC (e.g. rapid tests, urine strip) or in a private laboratory (e.g. culture, PCR, interferon-gamma release assays). Clipping of the claws, cleansing of eyes and ears, recording of body measurements, microchipping of the young cubs are some examples of other procedures that usually take place at that moment. If a particular case requires surgery (e.g. bullet wound exploration, dental surgery, etc.), the same is fulfilled at the time. Dental treatments (Figure 9) are the most common ones since the majority of rescue bears had their canines broke or deficiently pulled out by the Kalandars, resulting in posterior infection or necrosis of the root canal.

During all procedures, the animal must have an open intra-venous (IV) route and the anesthesia must be closely monitored as well as the animal's temperature[7].

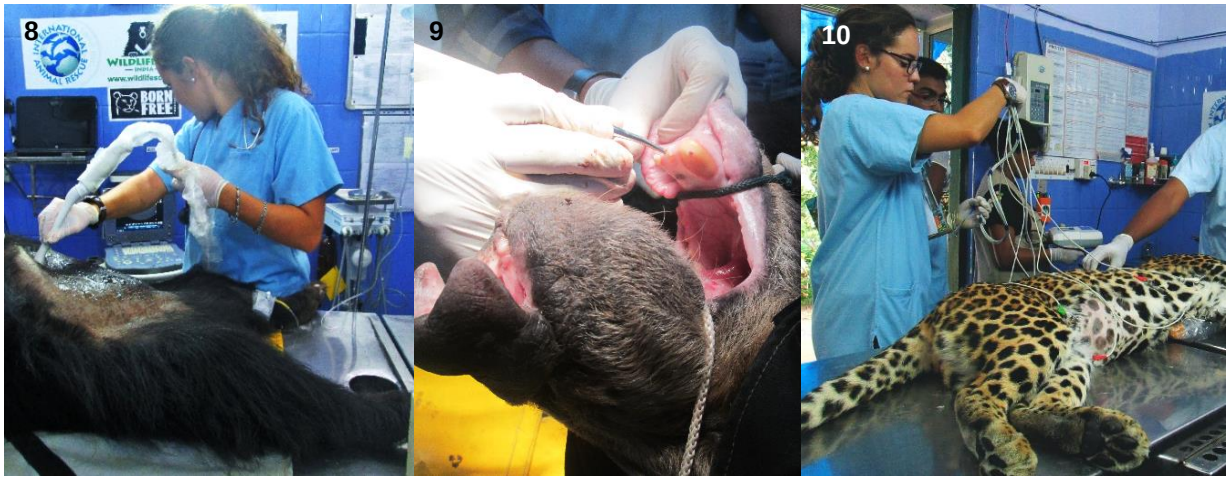
[7] Bears are able to use body fat to provide thermal insulation. These fatty deposits cause two issues when there is a need to sedate an animal: they hinder the administration of drugs by IM route and predispose to hyperthermia (von Hohendorff & Giacomini, 2014).

Besides bears, the author had the chance to be involved in other medical procedures with other animal species as leopards (Figure 10), lions, tigers, elephants and a python.

Figure 8- Ultrasonography examination on a sedated sloth bear (Original).

Figure 9- Dental surgery on a sedated sloth bear with a rotten root (Original).

Figure 10- Electrocardiography (ECG) performed on a leopard from the BBP Zoo (Original).



3. Laboratory Work: At a small laboratorial space (Figure 11) the veterinary staff was able to fulfill several simple procedures, as parasitological analysis of fecal samples and blood smears; centrifugation of blood samples, in order to obtain serum for the routine rapid tests for TB screening and microscopic analysis of lymph nodes aspirates. In order to carry out fecal parasitological analysis, the keepers are asked to keep the animals inside the enclosures, where they come to eat, after the morning feeding period (11am), and only to release them to the exterior after defecation. Fecal samples are collected to individual plastic jars, which are labeled and, in the same afternoon, the microscopic detection of parasite eggs is fulfilled (e.g. *Toxocara canis*, *Hymenolepis spp.*). Some animals excrete adult parasitic forms, which are macroscopically detected (e.g. round worm, tape worm). Although deworming medication is provided routinely in every enclosure, routine parasitological check-ups are very useful to keep a track of deworming drugs performance, of animal status, and, of course, to infer the parasite species most commonly found in these animals.

4. Daily Medical Treatments: The wound dressing of injured bears and tigers[8] was performed on a daily basis. In these animals, wounds are mostly caused by bullets, wire traps, and maggots. The four most routinely "patients" were a tiger with a paw maggot wound (Figure 12), a tiger with a torso injury, a bear with an infected gunshot wound and a wild bear that had fallen down a tree, after being pursued, threated and attacked by humans, and had several abrasions, cuts, a gunshot wound and a maggot wound (Figure 13).

[8] BBRC Veterinarians are also responsible for the care of 4 tigers that live in a different area.

Figure 11- Laboratorial area with microscope and centrifuge for basic lab work and sample process (Original).

Figure 13- Maggot wound on a tiger's forelimb extremity (Original).

Figure 12- Wild sloth bear with injuries caused by a tree fall and human attacks (Original).

5. Training/Conditioning: This activity was performed by the author on a daily basis using a clicker[9], having worked with 8 different bears. The use of positive reinforcement training (in this case, rewarding the animals with dates or honey) may enable many non-painful and minimally painful veterinary procedures such as inspection of teeth and feet, cleaning of wounds, injections, and blood drawing (Figures 14 & 15), without the need for anesthesia or physical restrain.

Figures 14 & 15 - Blood drawing attempt without the use of sedation. While individual nr.1 executes the trimming and disinfection of the animal's limb, individual nr.2 provides treats to the animal, as trained in many previous sessions (Original).

6. Environmental Enrichment Work: At least twice a week, besides the daily enrichment fruit distribution, some kind of enrichment project was put in action within the animals' enclosures (Figures 16 & 17). The author was strongly encouraged to participate in the same, either as a project designer, part of the construction team and/or as an observer and evaluator of the animal's behavior afterwards (Figures 18 & 19). This principle seeks to enhance the quality of captive animal care by identifying and providing the environmental stimuli necessary for optimal

[9] Clicker training is a type of training technique that uses a clicker device (little toy-like device that emits a "click" sound). Whenever the animal performs the trainers' requests correctly, the clicker will sound before the treat/reward is given. This way, the animal will know that it did not performed the order correctly if it does not hear that specific sound.

psychological and physiological well-being, preventing the onset of abnormal behaviors, also called stereotypic behaviors[10] which bears are very susceptible to develop. Some examples of this actions in bears are: head spinning, constantly walking from one side of the room to the other (pacing) and body and head rocking.

Figures 16 & 17- Construction of suspensive enrichment structure based on animals' "treat hunting" behaviour and posterior animals' reaction (Original);

Figures 18 & 19- Sloth bears enjoying environmental enrichment structures (Original).

7. Necropsies: Fortunately, not a single sloth bear died during the author's internship length. None the less, the author had the opportunity to perform several *post-mortem* examinations to deceased BBP Zoo animals (e.g. python and lion), wild birds and unfortunate stray animals from outside the reserve, victims of India's typical heavy traffic.

8. Rescue Missions: The author had the chance to accompany the head Veterinarian on two rescue missions: the "Circus' elephants rescue mission" (Figures 20 & 21) in October, where a rescue team from BBRC travelled to Tamil Nadu to inspect the health and living status of three circus' Indian elephants, in order to attempt a rescue later to Wildlife SOS Agra Rescue Facility; and the "Flood relief mission" (Figure 22) at the Chennai flooding in December, where the BBRC

[10] Repetitive, invariant behavior patterns with no obvious goal or function (von Hohendorff & Giacomini, 2014).

team went to Chennai for a one week period, after the flooding that occurred in the area from November to December, in order to rescue all kinds of animals in need and treat the injured ones, either at a rescue facility or directly in the streets of the city.

Figure 20- Indian elephant oral inspection (Original).

Figure 21- Treatment of nail fissures on an Indian elephant (Original).

Figure 22- Ruminant treatment durning the Chennai flooding at an Animal Rescue Centre (Original).

9. Individual Wildlife SOS (WSOS) Assignments: The author had several assignments given by the head veterinarian during her stay at BBRC, namely the development of a "Hygiene and Organization Check List", with all the necessary check points regarding the cleanness of the operation theatre before and after every clinical intervention; a "Processing Bear Bones Protocol", indicating the most proper way to process dead animal's bones, step by step, for learning and investigation purposes, creation of a "Conditioning Bear Evaluation" document, on which all trained bears had a proper file regarding its behavior and training achievements; improvement of the already existing "Bear Body Condition Score", development of aging scores based on dental X-ray evaluation and creation of awareness brochures regarding animal conditioning activities and environmental enrichment projects.

10. Oral Presentations: Frequently, the author, as well as other members of the veterinary staff, was encouraged to prepare oral presentations about any subject that interested her. The author prepared and presented several assays, namely: "Renal lesions in non-domestic felines", "Low cardiac output as physiological phenomenon in hibernating, free-ranging brown bears", "Portugal Wildlife", "Veterinary Medicine in Portugal", "Dates and its storage: the dangers for bears"[11] and "The C-reactive protein[12] (CRP) test in TB diagnosis in sloth bears".

[11]As the author noticed some stored bags of dates either with signs of rat bites (indicating its presence) or with rotten dates inside, she presented a small lecture regarding the dangers of leptospirosis and fungus in animals' food.
[12]The CRP is a substance produced by the liver in response to inflammation. High levels in the blood is a marker of any condition that causes inflammation. In this small study, 12 TB positive bears were analyzed for this protein but no changes were detected in the CRP levels.

Chapter III- Literature review

1. Etiology

1.1. Taxonomy and Description of the Genus

Mycobacteria belong to the genus *Mycobacterium*, which is the single genus within the family of Mycobacteriaceae, in the order Actinomycetales (Rastogi, Legrand & Sola, 2001; Isaza, 2003, Pfyffer, 2007; Olsen et al., 2010). Mycobacteria are aerobic, nonspore forming, nonmotile, slightly curved, slender, rod-shaped organisms and are 0.6-1.0 × 1.0-10 μm in size (Figure 23). Their cell walls high lipid content, that includes characteristic mycolic acids, excludes standard aniline dyes, so that once stained with special staining techniques, mycobacteria are resistant to discoloration. This property is termed acid fastness, so that mycobacteria are commonly referred to as acid-fast bacilli. In contrast, these microorganisms are not readily stained with the Gram method and are considered weakly gram – positive (Nolte & Metchock, 1995; Isaza, 2003; Pfyffer, 2007; Olsen et al., 2010; Grange, 2014).

The content of the cell wall enables the bacteria to be resistant to many antimicrobial agents, acidic and alkaline compounds and dehydration (Isaza, 2003; Miller, 2008). It also allows it to grow slowly inside macrophages and to be protected from phagocytosis, being an important virulence factor (Ernst, 2012).

Figure 23- Scanning electron micrograph of *Mycobacterium tuberculosis* organism (Source: National Institute of Allergy and Infectious Diseases [NIAID]. Licensed under Creative Commons Attribution 4.0: https://creativecommons.org/licenses/by/4.0/ via Flickr: https://www.flickr.com/photos/niaid/5149398656/?ytcheck=1).

Mycobacterial classification has relied on organisms' biochemical and phenotypic characteristics (Nolte & Metchock, 1995; Rastogi et al., 2001; Miller, 2008). Colony morphology varies among species, ranging from smooth to rough and from nonpigmented to pigmented (Pfyffer, 2007; Grange, 2014). Strains have also been identified within species using restriction fragment length

polymorphism (RFLP), spoligotyping, and DNA sequencing (Rastogi et al., 2001; Miller, 2008). Also, natural division occurs between slowly (>7 days to form visible colonies on solid medium) and relatively rapidly (≤7 days) growing species of mycobacteria (Pfyffer, 2007; Olsen et al., 2010; Grange, 2014).

Overall, Mycobacteria can be divided into three different groups: *Mycobacterium tuberculosis* complex, *Mycobacterium avium-intracellulare* complex and other mycobacteria species (Isaza, 2003; Backues, 2008; Olsen et al., 2010).

The term "tuberculosis", by convention, refers to infection with specific organisms belonging to the *Mycobacterium tuberculosis* complex, which includes *M. tuberculosis, M. bovis, M. caprae, M. africanum, M*. *microti, M. canetti and M. pinnipedii,* with *M. tuberculosis* and *M. bovis* being of the most importance (Montali et al., 2001; Pfyffer, 2007; Miller, 2008; Olsen et al., 2010).

Mycobacterium tuberculosis is the predominant disease-causing agent in captive sloth bears, as detected by culture and PCR (Arun et al., 2014; A.S. Arun, personal communication, April 20th, 2016).

1.2. Life Cycle

Tuberculosis by *M. tuberculosis* is, predominantly, an infection of the lower respiratory tract, mainly transmitted by the airborne route, in the form of droplet nuclei, that causes progressive pulmonary disease in mammals (Montali et al., 2001; Isaza, 2003; Maas et al., 2013; Fogel, 2015), though it can affect virtually every organ in the body (Ernst & Wolf, 2006).

After inhalation, the droplet nuclei reach the lung alveoli, where they are rapidly phagocytosed by alveolar macrophages (Schluger & Rom, 1998; Ernst & Wolf, 2006; Lin, Plessner, Voitenok & Flynn, 2007; Ernst, 2012; Sakamoto, 2012). After phagocytosis, mycobacteria organisms multiply within macrophages, leading to the death of the infected cells (Olsen et al., 2010; Ernst, 2012), and the expanding population of bacteria spreads to newly recruited macrophages and dendritic cells (DCs) (Ernst & Wolf, 2006; Lin et al., 2007). These macrophages are stimulated to produce pro-inflammatory cytokines (TNF, IL-12, IL-1, IL-6) and chemokines, driving the recruitment of more leukocytes to the site of infection, like neutrophils and monocytes, and begin to form the early granuloma (Lin et al., 2007; Ernst, 2012; Sakamoto, 2012). A subset of the bacteria also migrates to the local draining lymph node, transported by DCs, where they likely initiate an adaptive response by antigen presentation to naive lymphocytes that will proliferate, differentiate (to CD4+ and CD8+ T cells) and migrate back to the lungs to participate in the granuloma formation (Cooper & Flynn, 1995; Ernst & Wolf, 2006; Lin et al., 2007; Ernst, 2012; Sakamoto, 2012). CD4+ and CD8+ T cells are important in the immune response to *M. tuberculosis*, and function by secreting cytokines (including those that can activate macrophages, such as IFN-*γ* and TNF) (Lin et al., 2007). In addition, part of the bacterial population is believed to disseminate by the bloodstream to other peripheral organs, causing extra-pulmonary tuberculosis (Ernst & Wolf, 2006).

After the onset of adaptive immune responses, growth of tuberculosis in the lung is restricted, the progression of the disease is interrupted and most hosts become asymptomatic, not shedding bacteria, and are considered to have latent TB infection (Ernst & Wolf, 2006; Ernst, 2012). It has been suggested that the pathogenic bacteria lie latent in phagosomes inside macrophages, waiting for an immunocompromised condition of the host to grow quickly (Itagaki & Cho, 2013; Dietrich et al., 2015). This latency may also occur in the extra-pulmonary tuberculosis scenario (Ernst & Wolf, 2006).

In the majority of cases, the host adaptive immune response is sufficient to prevent active disease throughout life (Ernst & Wolf, 2006; Fogel, 2015). The animals that develop active disease will be the ones expelling infectious droplet nuclei to start the infection life cycle once more (Ernst & Wolf, 2006).

1.3. Transmission

An obligate step in all infectious diseases is transmission to new hosts (Ernst, 2012). TB transmission routes between animals are directly associated with the granulomas location in infected individuals. Pulmonary lesions generally lead to airborne transmission, in which bacteria are expelled (usually by coughing) from an individual with active disease and then inhaled by susceptible hosts, whereas mesenteric lymph-node lesions may lead to intestinal excretion of mycobacteria, that may be ingested by the next host (de Lisle et al., 2002; Backues, 2008; Mikota, 2008; Lécu & Ball, 2011; Ernst, 2012). Feces, urine, genital discharges, milk, and feed or water may contain contaminated droplets (Mikota, 2008). Horizontal transmission is the most significant and important mean of contamination (respiratory and alimentary routes) but pseudo-vertical transmission can potentially occur during nursing and grooming (Begins, 1999), and possibly vertical transmission through placental or umbilical infection (Kaneene & Pfeiffer, 2006).

As in many other infectious diseases, the transmission of TB is not uniform, and certain individuals cause far more secondary cases than do others. In particular, individuals with a form of TB termed cavitary TB are especially infectious ((Helke, Mankowskia & Manabe, 2006; Ernst, 2012).

2. Epidemiology

There are many reasons that can justify why sloth bears seem to be so susceptible to tuberculosis infection, the most important being obviously the close contact with infected humans, and the high prevalence of tuberculosis in the Indian subcontinent. The lack of positive TB findings in free ranging sloth bears, corroborates this theory (Renukaprasad et al., 2013; Arun et al., 2014; Kemmerer, 2015).

Next, some factors related with the epidemiology of tuberculosis infection in sloth bears are discussed (Figure 24).

2.1. Frequency and Prevalence

Between the years 2006 and 2016, out of 59 bear mortalities at BBRC, 42 were caused by *M. tuberculosis* infection (71%) (Table 4), and, by December of 2015, 32 live bears (16 males and 16 females), out of 86, were positive for TB (37%).

Table 4- Animal mortality at BBRC from 2006 to 2016 (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Year wise	Amount of resident bears	Total deaths	Deaths caused by TB infection
1st April 2006 - 31st March 2007	62	5	5
1st April 2007 - 31st March 2008	66	6	5
1st April 2008 - 31st March 2009	83	1	0
1st April 2009 - 31st March 2010	94	0	0
1st April 2010 - 31st March 2011	123	8	1
1st April 2011 - 31st March 2012	118	14	11
1st April 2012 - 31st March 2013	105	8	8
1st April 2013 - 31st March 2014	88	11	8
1st April 2014 - 31st March 2015	78	2	2
1st April 2015 - 31st March 2016	86	4	2
TOTAL	-	**59**	**42**

2.2. *Mycobacterium tuberculosis* Complex and Reported Hosts

The *M. tuberculosis* complex has a wide host range but, historically, maintenance hosts of the disease have been primarily ruminants and primates (Backues, 2008).

M. tuberculosis is the predominant cause of TB in humans, non-human primates, elephants (Isaza, 2003; Miller, 2008), domestic canines (Paul, 2014) and sloth bears (Arun et al., 2014), whereas *M. bovis* is the most common cause of TB in domestic cattle and wild mammals (Miller, 2008). *M. microti* is primarily found in small rodents and hyraxes but has also been isolated from llamas, pigs, and ferrets. *M. africanum* is a rare cause of TB in humans, cattle and pigs (Montali et al., 2001; Miller, 2008). *M. pinnipedii* has occurred in a variety of wild and captive sea lions and fur seals (Jurczynski et al., 2012).

M. bovis is widespread within domestic animals and has been extensively documented in both captive and free-ranging wildlife populations, being primarily an animal pathogen (Alexander et al., 2002; Isaza, 2003). In contrast, *M. tuberculosis* is considered mainly a human pathogen and has been reported only in domestic or wildlife species within captive settings, with close and prolonged contact with humans and does not appear to be common in free ranging animals (Montali et al., 2001; Alexander et al., 2002; Michel et al., 2003).

The list of hosts for each species of the *Mycobacterium* genus is resumed in Table 5.

Table 5- Tuberculosis Complex Mycobacteria and their reported hosts (Lécu & Ball, 2011).

***Mycobacterium tuberculosis* complex species**	**Major historical known host**	**Reported wild and zoo host**
M. tuberculosis	Human, non-human primates	Elephants, non-human primates, beisa oryx, addax, goats, birds, lowland tapirs, giraffes springboks, mongooses, rhinoceros, addra gazelles
M. bovis	Cattle (+ buffalo, bison)	All ruminants, badgers, possums, meerkats, big cats, canids, rodents, non-human primates, wild boars, elephants, camelids, rhinoceros, onagers, horses, birds
M. africanum	Humans	Cattle, swine, non-human primates
M. microti	Voles, camelids	New World monkeys, big cats
M. pinnipedi	Pinnipeds	Camels, tapirs, big cats
M. caprae	Goats, sheep, swine	Swine, cattle, wild boars, red deer, white tail deer, camels, bisons
M. canetti	Humans	?

2.3. The Carnivore Host

Mycobacterial infections are uncommon but serious diseases in domestic and wild species of carnivores (Parsons, Warren, Ottenhoff, van Pittius & van Helden, 2012; Montali et al., 2001; Backues, 2008; Collins, 2014), occurring sporadically from incidental infection through close contact with infected hosts or ingestion of infected animals (Backues, 2008; Miller, 2008; Arun et al., 2014). Although susceptibility to *Mycobacterium* spp. is not considered high in this family of mammals, infections caused by the three major groups of pathogenic mycobacteria have been documented. The severity of the disease and consequent sequelae make the presence of mycobacterial disease in carnivores an important clinical issue (Backues, 2008).

Cases of *M. tuberculosis* complex infections have been documented in domestic, captive and free-ranging carnivores of several families[13] (Backues, 2008; Paul, 2014), the majority of which have been caused by *Mycobacterium bovis* (Briones et al., 2000; Bruning-Fann et al., 2001; Ayele, Neill, Zinsstag, Weiss & Pavlik, 2004; Martín-Atance et al., 2006; Backues, 2008; Fico, Mariacher, Ciarrocca, Eleni, Franco & Battisti, 2015), including domestic felines, in which 90% of TB cases are associated with this species of mycobacteria (Paul, 2014). Domestic canines are an exception, with 75% of TB cases caused by *Mycobacterium tuberculosis*, which is most commonly transmitted by infected humans to animals (Martinho et al., 2013; Paul, 2014).

[13]Felidae, Canidae, Procyonidae, Mustelidae and others.

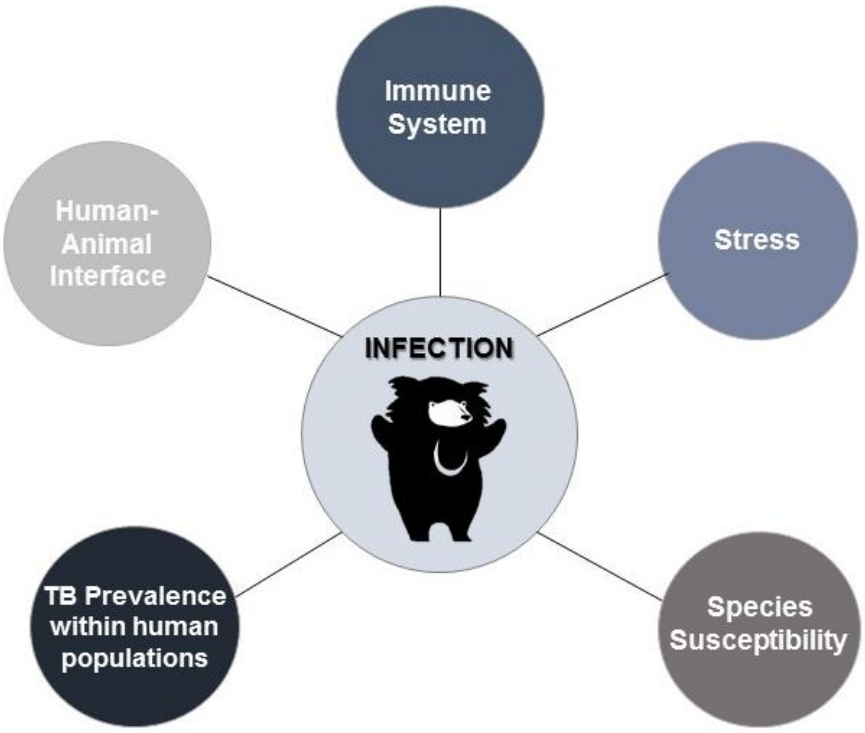
Sloth bears kept illegally in captivity are one of the most emblematic reported cases of human tuberculosis transmission in wildlife carnivores (Arun et al., 2014; Collins, 2014; Kemmerer, 2015), though, in this particular case, the close contact with humans may put them in the "domestic" animal category instead, making them directly comparable with the domestic canines previously mentioned.

In most countries, TB testing is not performed in this sort of animals as a standard, except when there is a high risk of exposure (EAZWV, Tuberculosis Working Group, 2010).

2.4. Susceptibility to Infection

Tuberculosis is a complex infection with many different factors involved that may influence the animal's susceptibility to the development of the disease (Figure 24). In the following chapter, five of the most important susceptibility factors in sloth bears will be analyzed in more detail.

Figure 24- Schematic representation of the risk factors associated with tuberculosis infection in sloth bears in the India subcontinent (Original). (Sloth bear image source: http://fightingferret.deviantart.com/art/Sloth-Bear-81282662).



2.4.1. Species Susceptibility

The course of the disease, as well as the occurrence of active and latent infection, varies among species, from extremely sensitive Old World monkeys to apparently more resistant equids (Lécu & Ball, 2011).

Several studies have provided evidence that susceptibility to develop TB is partially influenced by host genetic factors (Boomershine & Zwilling, 2000; Mikota, 2008; Stein, 2012), noticing

differences between humans and mice (Fortin, Abel, Casanova & Gros, 2007), between deers and various cattle species (Allen et al., 2010), and between Asian Old World non-human primates and African Old World non-human primates (Isaza, 2003).

It has also been reported that, in captivity, Himalayan black bears (*Selenarctos thibetanus*)[14] and sloth bears (*Melursus ursinus*) are found to be highly susceptible to tuberculosis infections (Rishikesava et al., 2008[15]) though it has not been a matter studied and analyzed in detail in literature. Interestingly, both dogs and bears (Canidae and Ursidae Families), which seem to be mostly affected by *M. tuberculosis,* and highly susceptible to it (Rishikesava, 2008; Martinho et al, 2013; Arun et al., 2014; Paul, 2014*)*, belong to the same superfamily in the Carnivora Order: Canoidea (McGraw-Hill Dictionary of Scientific & Technical Terms, 2003), which might suggest the existence of a genetic connection.

2.4.2. Immune System Status

Infection with mycobacterial organisms does not necessarily imply active disease. Several possible scenarios may result after exposure to an infectious source (Table 6) (Mikota, 2008). The outcome of mycobacterial infections and the progression of the disease are mainly determined by the balance between the microorganism and the host defense systems (Olsen et al., 2010; da Silva et al., 2015; Fogel, 2015). Hence, major changes in the immune status of the individual can potentiate TB activation or reactivation (Olsen et al., 2010; da Silva et al., 2015).

Once in the lung, mycobacteria organisms can have four potential fates (Figure 25): (1) the initial host response can be completely effective and eliminate all bacilli, thus, the animal will not develop tuberculosis in the future; (2) the organisms can begin to multiply and grow immediately after infection, causing clinical disease and formation of lesions, known as active tuberculosis, and the animal has the potential to infect others; (3) bacilli may become dormant after initial multiplication and never cause disease at all (latent infection); (4) the latent organisms may eventually begin to grow, with resultant clinical disease, known as reactivated tuberculosis, with formation of lesions (Schluger & Rom, 1998).

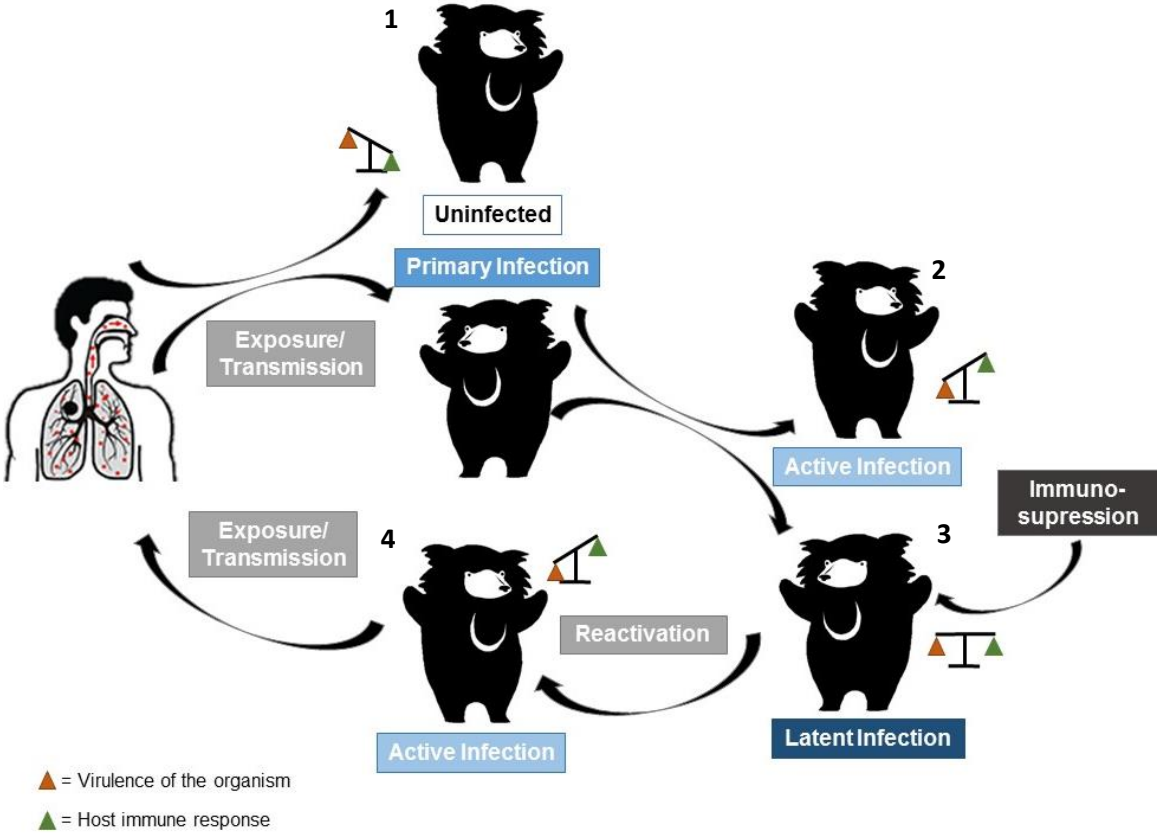
Table 6- Possible scenarios after exposure to active tuberculosis (TB) (adapted from Mikota, 2008).

Scenario	Pathogenesis	Tuberculin Skin Test Status (In Humans)
1	All bacteria are killed, resulting in no disease.	Negative
2	Bacteria multiply resulting in clinical disease (primary/ active TB)	Positive
3	Bacteria become dormant resulting in no clinical disease (latent TB)	Positive
4	Latent organisms reactivate and cause active disease.	Positive

[14] Rare subspecies of the Asiatic black bear (*Ursus thibetanus*);

[15] Arora, B.M. (2003). Indian Wildlife Diseases and Disorders. Association of Indian Zoo and Wildlife Veterinarians. (pp 381-382). Bareilly, India.

Figure 25- Schematic representation of TB transmission between captive sloth bears and humans. (Original). (Sloth bear image source: http://fightingferret.deviantart.com/art/Sloth-Bear-81282662; Human image source: https://upload.wikimedia.org/wikipedia/commons/7/73/Disease_transmission_sneezing.png).



The protective immunity against mycobacterial infections is mostly dependent on the activation of the cellular immune defense and, among the T cells responsive to mycobacteria, the CD4+ T cells are the most important (Olsen et al., 2010). That way, we can state that the protective immune response against mycobacteria is dependent on the interaction between host cells and CD4+ T cells. This was illustrated by using mice deficient in CD4+ T cells, that showed a much higher susceptibility to mycobacterial infections than mice lacking other types of T cells (Olsen et al., 2010), and by HIV infection in humans (Fogel, 2015), where decreased levels of CD4+ T cells result in the reactivation of tuberculosis (Olsen et al., 2010; da Silva et al., 2015). The reactivation of tuberculosis encountered in elderly people also suggests that a less efficient immune response can lead to the loss of control of a dormant infection (Olsen et al., 2010).

2.4.3. Stress

Chronic or long-term stress is known to have numerous adverse effects on health, some of which are mediated through actions on the immune system, as chronic stressors decrease circulating leukocyte numbers (Fowler 1986; Dhabhar, 2002; Dhabhar, 2014). It is now widely accepted that physical and psychological stress can modulate susceptibility to bacterial infection, such as

tuberculosis (Fowler, 1986; Boomershine & Zwilling, 2000; Mikota, 2008). The hypothalamic pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) mediate physiologic responses to stressors, where the HPA axis acts by producing a cascade of hormones resulting in increased production of glucocorticoids by the adrenals (Boomershine & Zwilling, 2000; Martin, 2009). Glucocorticoids were shown to suppress antimicrobial activity blood-derived macrophages and alveolar macrophages in humans (Boomershine & Zwilling, 2000). This way, treatment with glucocorticoids is also a well-known risk factor for reactivation TB (Ernst, 2012).

In animals, stressors like heat, cold, crowding, interactions with other animals, pain, parasitism, malnutrition, noise, habitat modification and restraint have been shown to alter the immune system, which may ultimately explain the physiological basis of disease-environment interactions (Kelley, 1980; Mikota, 2008; Martin, 2009; Kemmerer, 2015).

2.4.4. Human-Animal interface

The reservoirs for *M. tuberculosis* are infected humans so, naturally, infection by this organism occurs most frequently in animals that live in close and prolonged contact with humans with active TB, being one of the most frequently recorded infectious diseases of captive wildlife (Michalak, Austin, Diesel, Bacon, Zimmerman & Maslow, 1998; Alexander et al., 2002; Michel et al., 2003; Michel, Muller & Helden, 2010). It was even demonstrated, in an eleven-year study on twelve *M. tuberculosis* cases, in eight different species, of the National Zoological Gardens of South Africa, that the disease was more frequently transmitted by visitors to animals than between animals (Michel et al., 2003).

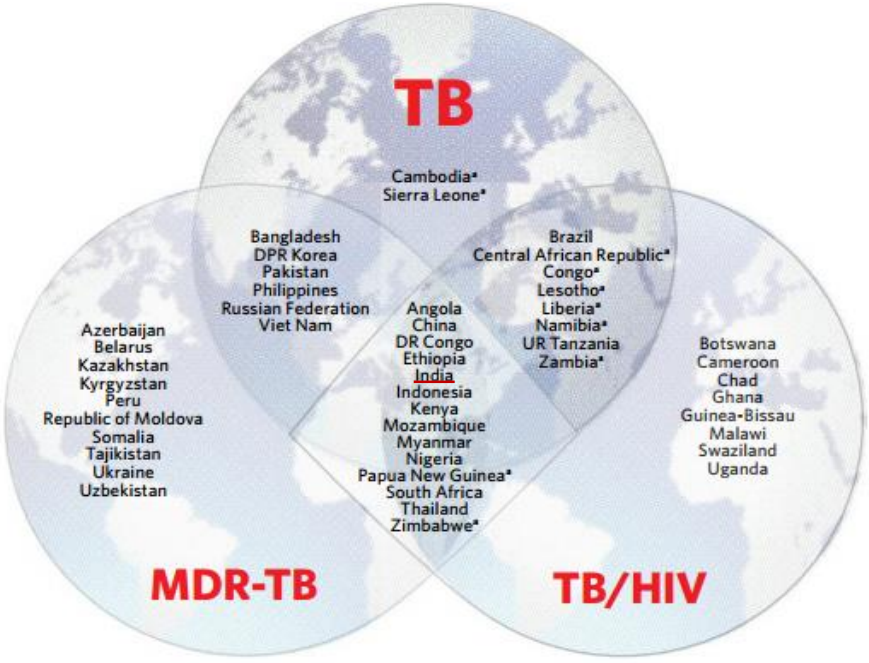
The domestic and routine Kalandar-bear relationship is comparable to the one humans normally share with their pets, in terms of contact and proximity, naturally empowering the transmission of several zoonosis, as TB (zooanthroponosis) (Arun et al., 2014). Similarly, many reports of canine TB highlight intimacy of contact between owners and their dogs as an important risk factor for TB transmission (Parsons et al., 2012; Martinho et al., 2013).

Also, in India, cattle co-exists closely with human populations, especially when we refer to communities with low economic status and/or somehow connected to the agriculture (i.e. animal traction). Cattle is the reservoir for *M. bovis* (Mikota, 2008; Lécu & Ball, 2011) which has the potential to infect humans and other animals, as bears (Collins, 2014; Fico et al., 2015). It is important to remember that captured “dancing bears” are also fed with cow’s milk by the Kalandars (Seshamani & Satyanarayan, 1997), most likely unpasteurized, being a potential hazard for mycobacterial infection. Although rescued “dancing (sloth) bears” are, to date, only found to be infected by *Mycobacterium tuberculosis* (A.S. Arun, personal communication, April 20th,2016), infection by other species of mycobacteria should always be taken into account when analyzing the epidemiology of tuberculosis infection in this species.

2.4.5. Prevalence of TB infection within human populations

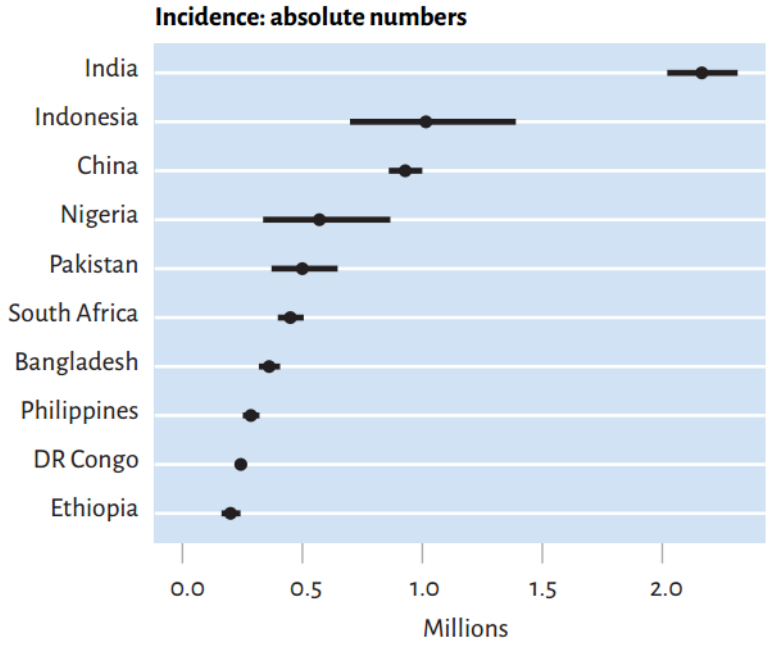
Logically, the risk of spillover of *M. tuberculosis* from humans to animals is higher in developing countries, burdened by high incidence rates of both human tuberculosis and human immunodeficiency virus (HIV) (Michel et al., 2003; Ernst & Wolf, 2006). Because T-lymphocyte-mediated immunity is essential for control of TB infection, the ongoing epidemic of HIV infection in regions with a high prevalence of tuberculosis is worsening an already severe problem. Moreover, the development of multiple drug resistance in *M. tuberculosis* (MDR-TB) has amplified the issues of tuberculosis treatment in many parts of the world (Ernst & Wolf, 2006) (Figure 26).
The six countries that stood out as having the largest number of incident cases of TB in 2015 were (in descending order) India, Indonesia, China, Nigeria, Pakistan and South Africa, keeping their 2014 status (Figure 27). Of these, China, India and Indonesia alone accounted for 45% of global cases in 2015 (WHO, 2016). As we can conclude, India is, nowadays, the country with the highest burden of TB (WHO, 2016), being also the third country in the world with the highest number of HIV infected people (Joint United Nations Programme on HIV/AIDS [UNAIDS], 2016) and the second one with the highest incidence of MDR-TB cases, following China (WHO, 2016). It is estimated that about 40% of the Indian population is infected with TB, the vast majority of whom have latent rather than active TB (WHO, 2015). Moreover, this situation takes bigger proportions when we are referring to communities with low economic incomes and very little access to health care, as the Kalandars. With this said, “dancing (sloth) bears” might just be one of the largest animal populations ever being in the riskiest scenario for TB infection.

Figure 26- Lists of countries belonging to the major three TB-related problematic categories and their areas of overlap (in Global Tuberculosis Report 2016 by WHO). India is in evidence in the picture in order to facilitate the analysis for the reader.



TB- Tuberculosis; MDR-TB- Multiple-drug resistance tuberculosis; TB/HIV- Tuberculosis infection within HIV positive people.

Figure 27- Estimated TB incidence: top-ten countries in 2014. The range shows the lower and upper bounds of the 95% uncertainty interval and the bullet marks the best estimate (in Global Tuberculosis Report 2015 by WHO).



2.5. Zoonotic Risk

In the case of sloth bears, the risk of animal-human transmission is naturally higher with daily handlers as vets and keepers (Backues, 2008: Lécu & Ball, 2011), and human-animal transmission is higher within poacher communities as the Kalandars (Arun et al., 2014; Kemmerer, 2015).

The potential for a human to acquire any mycobacterial disease from a captive wild animal (anthropozoonosis), as well as the other way around (zooanthroponosis), requires a combination of several events (Lécu & Ball, 2011). The host that is actively infected with a mycobacterial disease must be shedding and it must be in a form that has potential for infection, which is most likely in the form of aerosolized droplets from respiratory secretions[16]. Other potential sources of infection include urogenital or gastrointestinal shedding and the aerosolization of these materials during routine husbandry and cleaning (Dalovisio, Stetter & Mikota-Wells, 1992). An important zoonotic potential risk also exists in *post-mortem* examinations of infected animals (Lécu & Ball, 2011; USAHA, 2012).

[16] Captive sloth bears have the habit to "blow" through the enclosure's grids, which represents a potential hazard for human workers. It is advisable to wear masks, even when simply working within the enclosures, and not necessarily in direct contact with animals.

3. Pathogenesis

3.1. Generalities

Considerable evidence indicate that Mycobacteria have evolved specific mechanisms to manipulate their cellular niches for their own advantage, allowing them to survive in macrophages and to overcome innate and acquired host immune mechanisms (Boomershine & Zwilling, 2000; Ernst, 2012; Grange, 2014): (1) modulation of the trafficking and maturation of the phagosomes in which they reside, allowing them to evade lysosomal mechanisms of restriction, killing and degradation; (2) use of several virulence mechanisms to optimize their spread from cell to cell; (3) use of multiple mechanisms for inhibiting host cell apoptosis, which provides a prolonged survival time of infected cells and for a larger number of bacteria to accumulate in a given cell before they are released by cell death (Ernst, 2012).

As we can infer, these organisms are able to adapt to the distinct environments encountered during different stages of infection, by regulating their metabolism, protein expression and replication, in order to survive and eventually multiply and infect other animals (Olsen et al., 2010; Ernst, 2012; Dietrich et al., 2015). After the microorganisms get into the host macrophages and replicate intracellularly, eventually resulting in the lysis of their host macrophage, the released mycobacteria are ingested by newly arrived macrophages recruited from the bloodstream, resulting in further tubercular replication and macrophage lysis. As this cycle is repeated, primary lesions are formed (Boomershine & Zwilling, 2000; Olsen et al., 2010).

The pathogenicity of mycobacteria is a multifactorial phenomenon requiring the participation and cumulative effects of several components. The large range of antigens that the organism displays plays a key role in eliciting the various immune responses to the organism (Olsen et al., 2010; Grange, 2014). The virulence factors of *Mycobacterium tuberculosis* can generally be divided into 2 groups: proteins and cell wall components (Sakamoto, 2012).

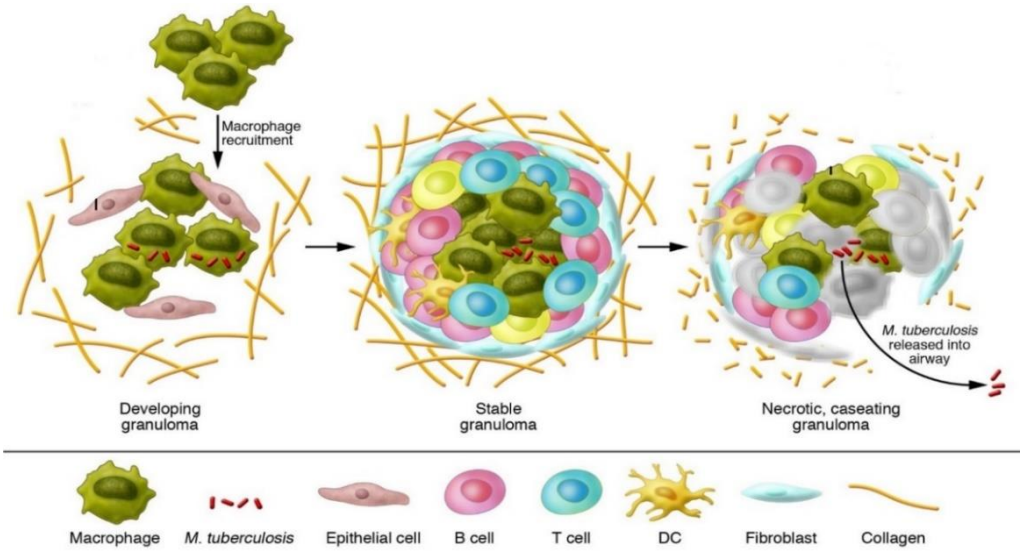
3.2. Lesions

The primary mycobacterial lesion is the granuloma (Figure 28), a chronic inflammatory response which is found in affected tissues and the draining lymph nodes (Boomershine & Zwilling, 2000; Lin et al., 2007; Olsen et al., 2010; Ernst, 2012; Sakamoto, 2012). This organized collection of immune cells is maintained by the persistent presence of antigen (Lin et al., 2007). It consists of a central region of infected macrophages surrounded by epithelioid macrophages, foam cells and occasional multinucleated giant cells of the Langhans type, with peripheral recruited lymphocytes (both CD4+ and CD8+ T cells, and B cells) and a fibrous capsule (Boomershine & Zwilling, 2000; Lin et al., 2007; Olsen et al., 2010; Ernst, 2012; Sakamoto, 2012). The lack of oxygen and the cytotoxic effects of activated macrophages lead to necrosis of the granuloma center (Boomershine & Zwilling, 2000; Sakamoto, 2012). The high lipid and protein content of the dead

macrophages result in a caseous appearance, grossly and histologically (Sakamoto, 2012). The bacteria appear to survive within macrophages and in the caseous center of the granuloma (Lin et al., 2007). Although the granuloma contains the mycobacteria and prevents its dissemination to other areas, it also provides a site where the bacteria population can be “hidden” from the immune system (Olsen et al., 2010; Sakamoto, 2012).

Granulomas, however, are not exclusive (or independently diagnostic) of *M. tuberculosis* complex infection, as they can also form around foreign bodies and can be found in a number of other pathologies such as sarcoidosis. A granuloma caused by *M. tuberculosis* is actually called a *tubercle*. The outcome of the tubercle formation is dependent on how effective the host’s immune system is at containing the infection (Ernst, 2012; Sakamoto, 2012).

Figure 28- Schematic representation of granuloma formation (Adapted from Salgame, 2011).



Pulmonary cavitation (cavitary tuberculosis) is a characteristic feature of secondary tuberculosis, and refers to the occurrence of gas-filled spaces, when necrosis involves the wall of an airway and the semi-liquid necrotic material is discharged into the bronchial tree (usually coughed out), leaving an empty space where it used to be (Gadkowski & Stout, 2008). This phenomenon was previously recorded in humans, mice, guinea pigs and non-human primates, and it is probably related to a pre-existing hypersensitivity to *M. tuberculosis,* from a prior primary infection, meaning that the host immunological reaction to the organism is implicated in the development of TB cavities (Helke et al., 2006). Multiple studies have revealed that humans co-infected with TB and HIV have a lower frequency of pulmonary cavitation, as well as the existence of a linear correlation between the number of circulating CD4+ T cells and the frequency of cavitary TB (Ernst, 2012).

3.2.1. Lesions Distribution

The development and distribution of lesions will depend on the route by which the animal was infected, if secondary spread of infection has occurred within the body, virulence of the organism,

and host susceptibility (de Lisle et al., 2002; Backues, 2008). Animals with lesions restricted to the thoracic cavity are assumed to be infected by inhalation of aerosols of the tubercle bacilli, while animals with lesions located in mesenteric lymph nodes, are thought to have acquired the infection either by ingestion, or by dissemination of a primary pulmonary infection to other organs (de Lisle et al., 2002; Ernst & Wolf, 2006). Also, the location of the lesion may indicate the probable mean of spread of infection (de Lisle et al., 2002).

3.2.2. Macroscopic lesions

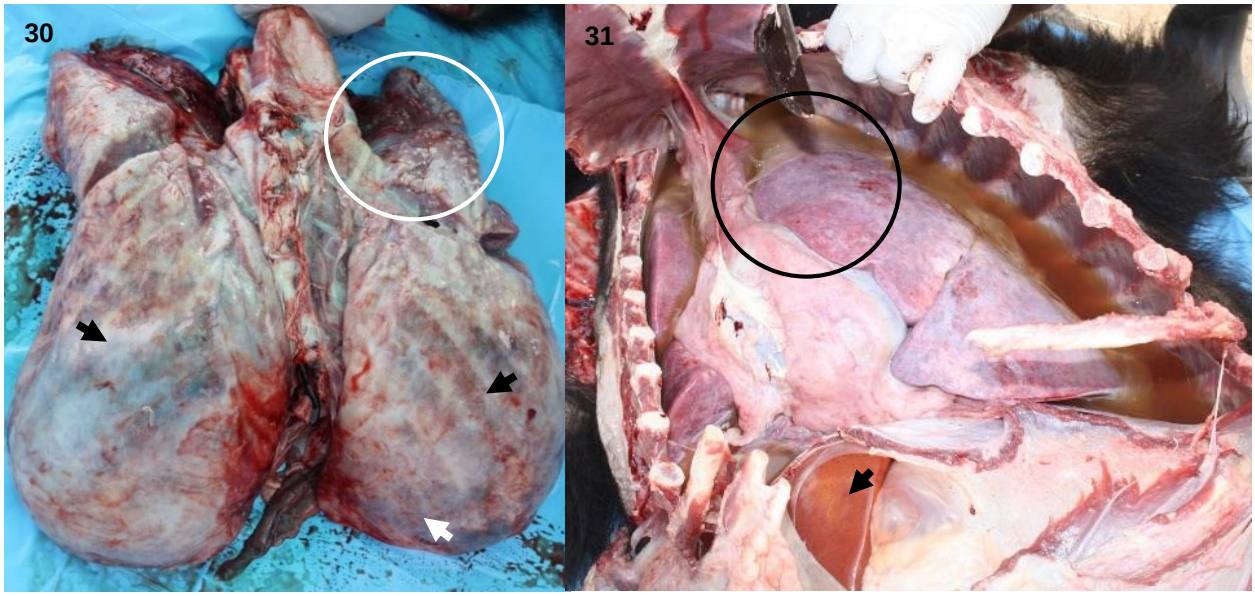
At *post-mortem* examination, generalized emaciation is evident (Figure 29). Lungs are the most affected organs and, once the thoracic cavity is opened, granulomatous pneumonia can be observed, as well as lung consolidation, inter lobular adhesions, rib cage impressions (Figure 30), pleural adhesions, and signs of congestion and hemorrhage (Figure 31) (Mehrotra, 1999; A.S. Arun, personal communication, December 29th, 2011). The alveolar spaces are usually filled with dense suppurative fibrin-purulent exudates (Figures 32 & 33) and nodules (tubercles) of various sizes are present (Mehrotra, 1999; Rishikesava et al., 2008; Fefar et al, 2012). In some cases, fluid in the thoracic cavity, indicative of hydrothorax, can be found (Figure 31) (A.S. Arun, personal communication, December 29th, 2011).

The mucous membranes are, in many cases, pale and icteric with the subcutaneous tissues presenting a yellowish coloration (Fefar et al., 2012). The abdominal cavity is usually filled with ascetic fluid and the peritoneal surface of diaphragm, peritoneum, and spleen might display caseative nodules in case of bacterial dissemination (Mehrotra, 1999; A.S. Arun, personal communication, December 29th, 2011; Fefar et al., 2012). Extra-pulmonary tuberculosis may occur but is not a common case. It is also possible to observe hemorrhagic enteritis, hepatitis, kidney congestion, and enlargement of the spleen, liver and mesenteric and hilar lymph nodes (Mehrotra, 1999; A.S. Arun, personal communication, December 29th, 2011).

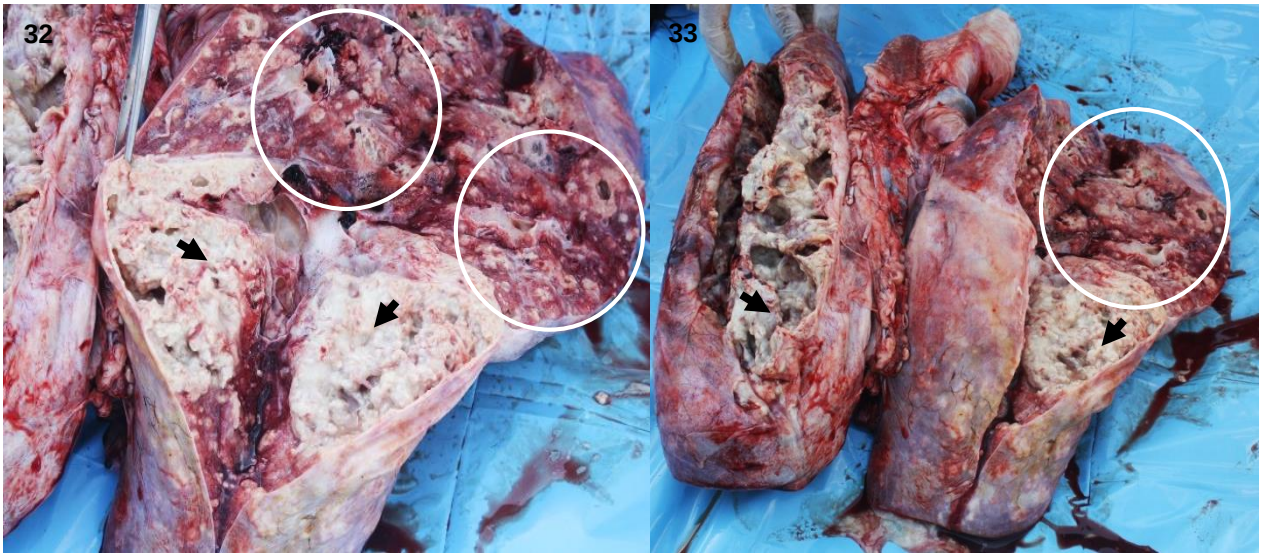
Figure 29- Emaciation of diseased TB positive sloth bear (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Figure 30- Enlarged TB positive sloth bear's lung. Rib cage impressions (black arrows), tuberculous nodules (white circle) and areas of congestion (white arrow) can be seen. (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Figure 31- TB positive sloth bear's lungs with areas of congestion (black circle), presence of hydrothorax and icteric liver (black arrow) (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).



Figures 32 & 33- 17-year-old male TB positive sloth bear's lungs. Absence of normal parenchyma and alveolar structure. Several nodules (white circles) and purulent thick material (black arrows) are present. (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).



Sloth bears' necropsy findings are very similar to the ones found in dogs infected by *M. tuberculosis* (Martinho et al., 2013).

3.2.3. Microscopic lesions

The histopathological exam of various affected organs may reveal the presence of multifocal granulomas, areas of focal necrosis and infiltration of chronic inflammatory cells with predominance of macrophages and epithelioid cells (Fefar et al., 2012). In Table 7, the specific lesions present in each organ are described in greater detail.

Table 7- Organ specific lesions found in sloth bears *post-mortem* examination at BBRC (A.S. Arun, personal communication, December 29th, 2011).

Organ	Lesion
Lung	❖ Ruptured alveolar structures; ❖ Infiltration of alveolar structures with epithelioid macrophages, Langham's giant cells, lymphocytes, plasma cells, and fibroblasts; ❖ Granulomatous lesions with caseation and necrosis.
Liver	❖ Cirrhosis; ❖ Hemorrhages; ❖ Fibrosis; ❖ Sinusoidal congestion; ❖ Inflammatory cells infiltration; ❖ Sequestration of red blood cells (RBC) in lobular spaces.
Heart	❖ Disruption of myofibrils; ❖ Interfibrillar RBC pockets.
Pancreas	❖ Infiltration of inflammatory cells;
Stomach	❖ Caseated epithelial cell granuloma; ❖ Hemorrhages.
Intestine	❖ Necrosis; ❖ Infiltration of inflammatory cells; ❖ Ruptured villi.
Kidney	❖ Granulomatous lesions with surrounding histiocytes; ❖ Interstitial nephritis; ❖ Congestion; ❖ Necrosis.
Adrenal	❖ Granulomatous lesions; ❖ Infiltration of lymphocytes.
Lymph Nodes	❖ Granulomatous lesions with caseation and necrosis; ❖ Fibrosis; ❖ Giant cells infiltration; ❖ Hemorrhages; ❖ Hyalinization.

3.3. Clinical Signs

A number of significant challenges arise in detecting tuberculosis in captive and free-ranging wildlife. Even in susceptible species, the majority of infected animals show no clinical signs indicative of disease (de Lisle et al., 2002; Backues, 2008).

Tuberculosis is characteristically a chronic wasting disease; thus the term “consumption” was used to describe the disease in humans in the early twentieth century (Mikota, 2008). In mammals, the most common sign is chronic weight loss (de Lisle et al., 2002; Mikota, 2008) and sloth bears are no exception, also manifesting signs as anorexia, prostration and general weakness (Mehrotra, 1999; Arun et al., 2014; A.S. Arun, personal communication, April 20th, 2016).

Chronologically, initial symptoms manifested by sloth bears, may resume themselves as simple gastritis, sometimes coupled with enteritis, and anorexia, evolving then to weakness weight loss and emaciation. Respiratory signs like cough, nasal discharge or dyspnea have been reported but appear to be uncommon, usually manifesting in the final stages of disease (A.S. Arun, personal communication, April 20th, 2016). Sick animals will also show decrease of activity and will tendentiously isolate themselves from the rest of the group (de Lisle et al., 2002; Rishikesava et al., 2008; Renukaprasad et al., 2013; A.S. Arun, personal communication, April 20th, 2016). All the previously mentioned symptoms are very similar to the ones found in domestic canines (Martinho et al., 2013).

In the beginning of the symptomatic phase of infection, sloth bears will start to reject the porridge, feeding only, for around four to five weeks, of what it is generically called ‘enrichment food’ (special and tastier items like fruits, dates and honey). After that, they will completely reject solid food, surviving only on water during the maximum period of 20-30 days (A.S. Arun, personal communication, April 20th, 2016).

The emaciation and loss of body condition will increase each day. During this period, bears might exhibit digestive symptoms as vomiting, foul smell, diarrhea and signs of abdominal distention and discomfort. Their breathing will slowly, but noticeable, get faster and shallowed, evolving to dyspnea and abnormal posture (head held high and extended neck). In the end, the animal is completely emaciated, dull and weak (A.S. Arun, personal communication, April 20th, 2016).

The progressive nature of the disease and the animal’s failure to show clinical signs until the disease becomes advanced, often lead to cases being diagnosed at necropsy, which represents a major threat to public health (Iallegio, 1997; Mehrotra, 1999; de Lisle et al., 2002; Backues, 2008; Miller, 2008; Arun et al., 2014). Therefore, a proactive quarantine and routine screening program should be developed for each zoological collection or wildlife sanctuary, housing susceptible species (Miller, 2008; Rishikesava et al., 2008; Lécu & Ball, 2011; USAHA, 2012).

4. Diagnosis

One of the essential issues of managing wildlife TB is the availability of diagnostic assays, which is often limited to those developed for domestic animals and humans (Maas et al., 2013; Arun et al., 2014). On a daily basis, wildlife species are tested for TB for various purposes, often with diagnostic assays that are accepted due to a lack of a better alternative (Maas et al., 2013). Nevertheless, it is important to keep in mind that most tests are not validated in wild animal species and, especially those based on immunologic response, may show significant variability among species (Miller, 2008; Maas et al., 2013), being hardly transposed from one species to another (Lécu & Ball, 2011). In sloth bears, as in the case of many other wild species, there is critical lack of accredited tests for tuberculosis screening (Rishikesava et al., 2008; Arun et al, 2013; Arun et al., 2014).

An increasing number of studies have been dedicated to the development of diagnostic assays for specific wildlife species, resulting in assay prototypes and partially or fully validated assays (Maas et al., 2013). Ascertaining the diagnostic sensitivity and specificity of the various *ante-mortem* tuberculosis screening tests will help in the early diagnosis and posterior treatment monitoring of this disease (Miller, 2008; Rishikesava et al., 2008; Arun et al., 2014).

As said, no *ante-mortem* test is 100% reliable for detecting TB in wild animals (Iallegio, 1997; de Lisle et al., 2002; Miller, 2008; Lécu & Ball, 2014). All of the procedures have limitations, but also the potential for determining, not only the existence of tuberculosis within a population, but the prevalence of infection in that population as well (de Lisle et al., 2002). Most of the time, an indirect test alone is meaningless if it is not combined with clinical examination, direct screening and another indirect assay (Miller, 2008; Lécu & Ball, 2014). Combinations of tests always improve the accuracy of TB diagnosis, as long as parallel tests that are independent of each are chosen (Guillerm, Usdin & Arkinstall, 2006; Miller, 2008; Lécu & Ball, 2014).

4.1. Unspecific Diagnosis

4.1.1. Clinical signs

As mentioned before, clinical signs are rarely seen before death in these animals (Montali et al., 2001; Backues, 2008; Renukaprasad et al., 2013; Arun et al., 2014). When respiratory signs, like cough and dyspnea, are noticed, it is always in a very late and irreversible stage of the pulmonary form of disease (Backues, 2008; Lécu & Ball, 2011; A.S. Arun, personal communication, April 20th, 2016). The first symptoms sloth bears exhibit usually involve the digestive system, evolving later to inappetance, anorexia, loss of body weight and respiratory symptoms (Renukaprasad et al., 2013; A.S. Arun, personal communication, April 20th, 2016).

4.1.2. Imaging

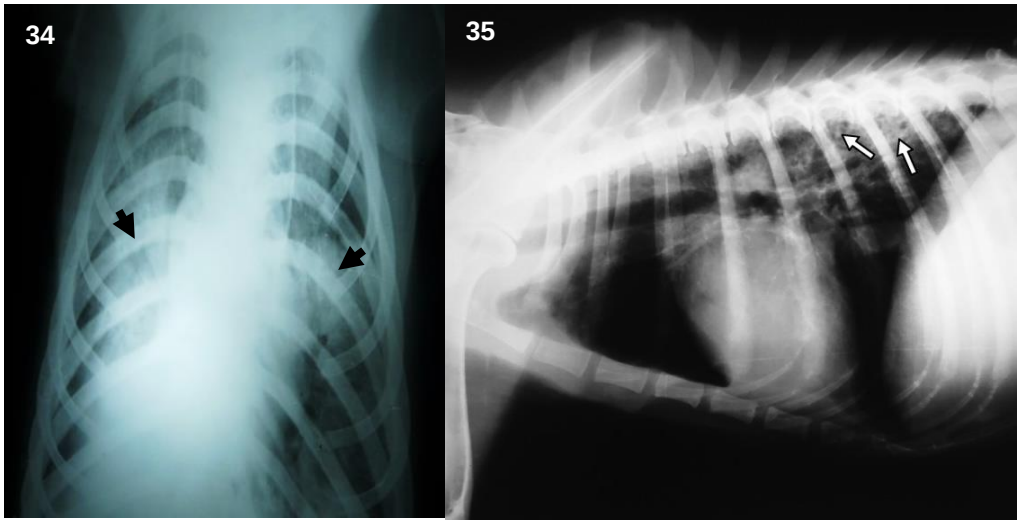
Different imaging techniques can also play a role in TB diagnosis. Unfortunately, they only provide information regarding the location and aspect of the lesions (Lécu & Ball, 2011). Radiography performance may be restricted by the limitations of animal species, like the size of the thoracic cage, the lack of reference images and the fact that lesions might be too subtle to be detected (Lécu & Ball, 2011). High-frequency ultrasonography, computed tomography (CT), magnetic resonance imagery (MRI) and even positron emission tomography (PET) scans could also be useful to detect granulomas or lesions in non-palpable lymph nodes when they are located in the thorax, but these techniques are frequently unavailable in zoological or rescue centre settings (Lécu & Riquelme, 2008; Lécu & Ball, 2011).

In sloth bears, radiography is the only imaging technique used (Figure 34) (Renukaprasad et al., 2013; Renukaprasad et al., 2014). Signs of bacterial pneumonia, cavitary lesions, multi-focal nodules and calcification may be found (Backues, 2008; Renukaprasad et al., 2013; A.S. Arun, personal communication, April 20th, 2016).

Calcified lesions are the ones that are better detected by radiography, although they do not always occur, being an inconsistent sign (EAZWV, Tuberculosis Working Group, 2010; A.S. Arun, personal communication, April 20th, 2016). On top of that, a lot of species do not show any calcified lesions at all, so this is not a routine exam in any animal species (EAZWV, Tuberculosis Working Group, 2010). Bears and dogs (Figure 35) are some of the species that display these lesions inconsistently (Martinho et al., 2013; A.S. Arun, personal communication, April 20th, 2016).

Figure 34- TB positive sloth bear thoracic X-ray. Some radiopaque areas (black arrows) indicate a possible presence of calcified lesions in the lungs (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Figure 35- Lateral thoracic radiograph of a dog with disseminated *Mycobacterium tuberculosis* infection. Note diffuse radiopaque images (white arrows) in dorsal and caudal lung lobes, suggesting lung consolidation and granuloma formation. (Source: Martinho et al., 2013, ©*The American Society of Tropical Medicine and Hygiene).*



4.1.3. Clinical Pathology (Hemathology and Serum Chemistry)

Clinical pathology cannot provide a definitive diagnosis of TB as there are no hematological or serum chemical abnormalities unique to mycobacteriosis in sloth bears, or carnivores in general (Backues, 2008; Arun et al., 2014; A.S. Arun, personal communication, April 20th, 2016). Blood count changes are generally resumed to nonspecific and inconsistent signs of inflammatory disease: anemia, lymphopenia and neutrophilic leukocytosis (Backues, 2008; Rishikesava et al., 2008; Martinho et al., 2013; Arun et al. 2014; A.S. Arun, personal communication, April 20th, 2016). Potentially serum chemistry profile abnormalities consist primarily of hypoalbuminemia and hypergammaglobulinemia (Iallegio, 1997; Backues, 2008). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels may be elevated (Iallegio, 1997; A.S. Arun, personal communication, April 20th, 2016).

4.1.4. Gross Pathology

A presumptive diagnosis of tuberculosis is often made on the basis of finding characteristic macroscopic lesions (de Lisle et al., 2002). In sloth bears, it would be the typical granulomatous appearance of tuberculous lesions, especially and mostly in the lungs (Rishikesava et al., 2008; Arun et al., 2014). It is also necessary to examine the gastrointestinal system and lymph nodes related to affected organs (Backues, 2008; A.S. Arun, personal communication, April 20th, 2016). The sensitivity of this method is naturally higher in advanced stages of the disease (Maas et al., 2013).
Other pathological changes were stated in the chapter “3.2.2. Macroscopic Lesions”

4.1.5. Histopathology[17]

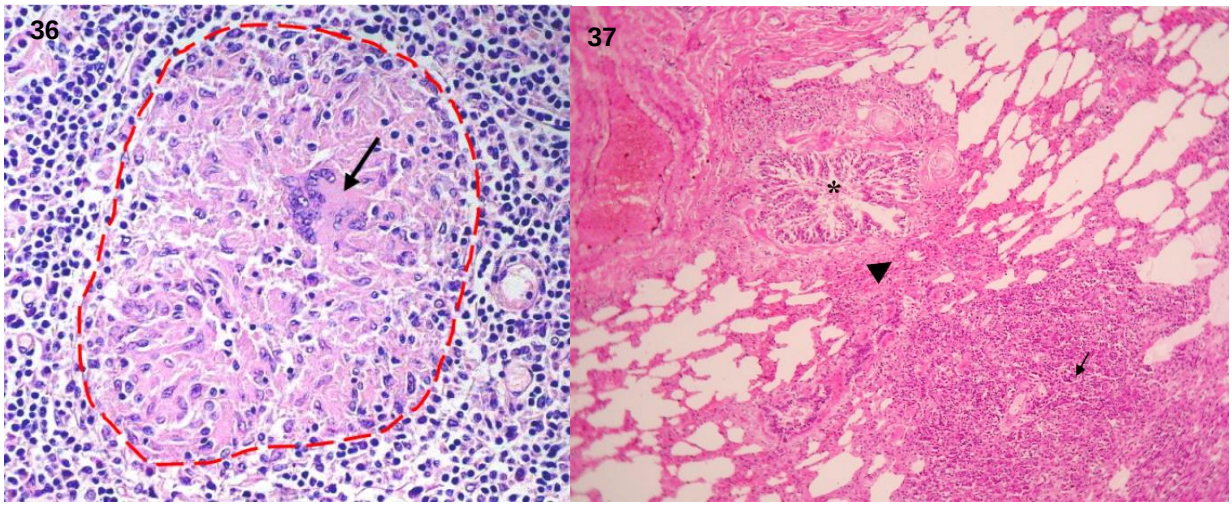
The examination of histological sections of suspect tuberculous lesions (e.g. in organs, lymph nodes) is a reliable diagnostic method, after suspicious macroscopic lesions are detected at *post-mortem* examination (de Lisle et al., 2002; Backues, 2008; Arun, 2013). Histopathology can be used not only to identify suspect cases of tuberculosis, but also to exclude such a diagnosis, as lesions caused by helminth parasites, some bacteria genera and some fungi, may be macroscopically mistaken by lesions caused by mycobacteria (de Lisle et al., 2002).
One of the limitations of histopathology is that the tissue damage in lesions caused by the *M. tuberculosis* complex are often indistinguishable from each other (de Lisle et al., 2002).
Presence of acid-fast organisms, giant cells, fibrous encapsulation, mineralization, and necrosis with neutrophil infiltration, associated with the granuloma formation (Figures 36 & 37), are some indicative histological features of TB lesions (de Lisle et al., 2002).

[17] This method was suited for both unspecific and direct diagnostic categories, as it is also possible to identify the bacilli. It ended up in the first group, considering that the main goal of this method is not to detect the acid-fact bacilli itself but tissue abnormalities that are indicate of its presence and action.

Tissue samples for histology are normally formalin-fixed, embedded in paraffin wax, sectioned and stained with Hematoxylin-Eosin (HE) and Ziehl-Neelsen (ZN) (Fico et al., 2015).

Figure 36- Granuloma (hematoxylin and eosin stain) in human lung. The red outline highlights the distinct structure of the granuloma, caused by immune cells bunching together to surround the pathogenic organism The arrow shows a multinucleated giant cell formed by multiple macrophages that have fused together (Source: https://micro2tele.files.wordpress.com/2012/03/granuloma.png).

Figure 37- Photomicrograph of a lung from a dog infected by *Mycobacterium tuberculosis*, showing granulomatous reaction with central necrosis, presence of epithelioid cells and fibroblasts in the middle zone (small arrow). Note proximity of granuloma (large arrow) with bronchial tree (*) indicating active tuberculosis (TB) infection (H&E stain, ×200). (Source: Martinho et al., 2013, ©*The American Society of Tropical Medicine and Hygiene).*



4.2. Direct assays

Diagnostic tests that identify the presence of *Mycobacterium* organisms are considered the most definitive methods of diagnosing infection (Miller, 2008; Lécu & Ball, 2011; Maas et al., 2013). Direct identification of the agent may be performed by microscopic demonstration of acid-fast bacilli in various samples, bacterial culture and nucleic acid amplification techniques (Abebe et al., 2007; Backues, 2008; Miller, 2008; Maas et al., 2013).

4.2.1. Culture

Isolation of mycobacterial organisms by culture remains the gold standard for both diagnosis and drug sensitivity testing (Guillerm et al., 2006; Arun et al., 2014; Lécu & Ball, 2014) and is, as such, used for comparison when validating new assays (Maas et al., 2013). It may also be useful for differentiating different species of *Mycobacterium* by colony morphology, growth rate, biochemical characteristics, and microscopy (Pfyffer, 2007; Grange, 2014).

Culture is unlikely to yield a false-positive, displaying a high specificity throughout different studies, and its sensitivity is generally estimated rather high in some species, especially in the presence of lesions in the cultured tissues (Mikota, 2008; Maas et al., 2013), which indicates that sensitivity is likely to be higher in *post-mortem* samples. In elephants, this method's sensitivity is considered very low *ante-mortem* (using a trunk wash, similar to sputum and Broncho-Alveolar Lavage [BAL] in sloth bears) with many infected animals failing to be identified (Mikota, 2008). In a culture, it is possible to detect bacilli loads above 10^1-10^2 bacilli /mL (Mikota, 2008; Lécu & Ball, 2011).

Culture of mycobacteria can be performed using different culture systems, though the most widely used medium is Löwestein-Jensen (LJ) (Figures 38 & 39), where bacteria are cultured aerobically at 37ºC (Miller 2008; Maas et al., 2013; Grange, 2014). Improved culture methods associated with automated micro bacteriologic detection systems such as the BACTEC®[18], Septi-Chek®[19] and mycobacterial growth indicator tube (MGITs®[20]), have the potential to decrease the time of growth detection and increase the rate of recovery (Guillerm et al., 2006; Miller, 2008; Lécu & Ball, 2014).

Though culture is considered the "gold standard", it has some important limitations (Miller, 2008). The amount of bacilli detected in biological *ante-mortem* samples (i.e. sputum) may be affected by various factors, like highly calcified lesions (Capuano et al., 2003), intermittent shedding, site of infection and difficulty of obtaining *ante-mortem* samples from some species (Miller, 2008; Lécu & Ball, 2014). Even in humans, 15 to 20% cases with active pulmonary TB are not even confirmed by any culture (Lécu & Ball, 2011), as the bacilli may be protected in lung cavities (cavitation) or not present in a particular sputum sample, or even be lost in the decontamination treatment required to process sputum for mycobacterial culture (Frieden et al., 2003; Guillerm et al., 2006). Therefore, while positive culture provides evidence of disease, negative culture results may not rule out infection in exposed or suspect animals (Lécu & Ball, 2011; Maslow & Mikota, 2015). A further disadvantage is that Mycobacteria are slow growing and results take around 8 weeks (6-12 weeks) to be obtained in Löwestein-Jensen medium (Iallegio, 1997; Vargas et al., 2005; Mikota, 2008; Miller, 2008; Maas et al., 2013).

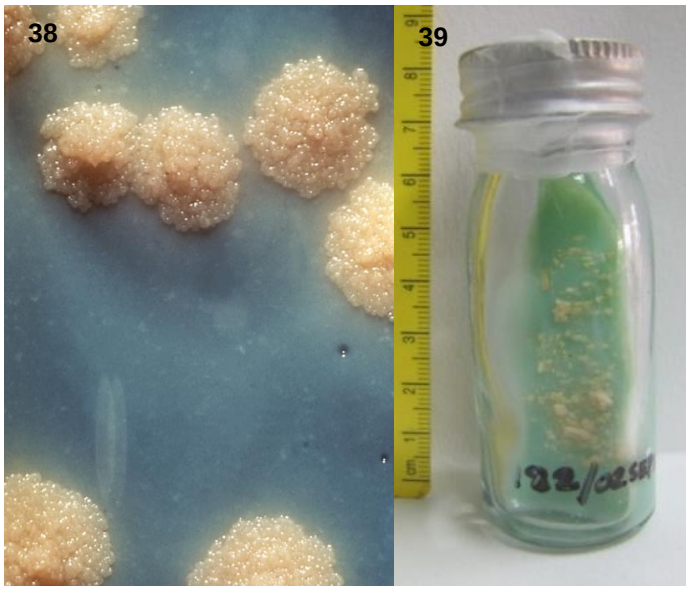
As mentioned, culture may also be useful for differentiating species within *Mycobacterium* genus, not only by the macroscopic and microscopic differences between colonies but also by the fact that the actual isolation techniques differ among species. For instance, *M. bovis* grows poorly, or not at all, on standard glycerol-containing Löwenstein-Jensen medium as used for isolation of *M. tuberculosis*, but its growth is stimulated by sodium pyruvate (Grange, Yates & de Kantor, 1996). In captive sloth bears, samples for culture are send to human medicine laboratories. Until now, only positive results for *M. tuberculosis* were given upon colony development in an adequate

[18] BD Diagnostics, USA;
[19] BD Diagnostics, USA;
[20] BD Diagnostics, USA;

medium (read previous paragraph) and its posterior characterization (Renukaprasad et al., 2013; Arun et al., 2014).

Figure 38- *Mycobacterium tuberculosis* colonies in Löwestein-Jensen medium from a human sputum sample (Source: https://upload.wikimedia.org/wikipedia/commons/0/0a/TB_Culture.jpg).

Figure 39- *Mycobacterium tuberculosis* colonies in Löwestein-Jensen medium from a sloth bear sputum sample (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).



4.2.2. Acid-Fast Staining Microscopy (ZN Staining)

Direct staining of sample materials with the Ziehl-Neelsen method (ZN) may provide presumptive identification as acid-fast bacteria (Figures 40 & 41) (Backues, 2008; Mikota, 2008; Miller, 2008; Lécu & Ball, 2011; Arun et al., 2014), being the fastest, easiest and least expensive method of TB direct diagnosis (Mikota. 2008; Rishikesava et al., 2008; Somoskovi, Gutierrez & Salfinger, 2008).

Although the specificity is excellent (all mycobacterial species are acid-fast), the sensitivity is not optimal, and the method is unable to distinguish among species within the *Mycobacterium tuberculosis* complex (Somoskovi et al., 2008). Furthermore, there are also non-mycobacterial organisms, such as *Nocardia*, that also share the acid-fast resistant property, reacting to the ZN dyes as well (Miller, 2008; Lécu & Ball, 2011).

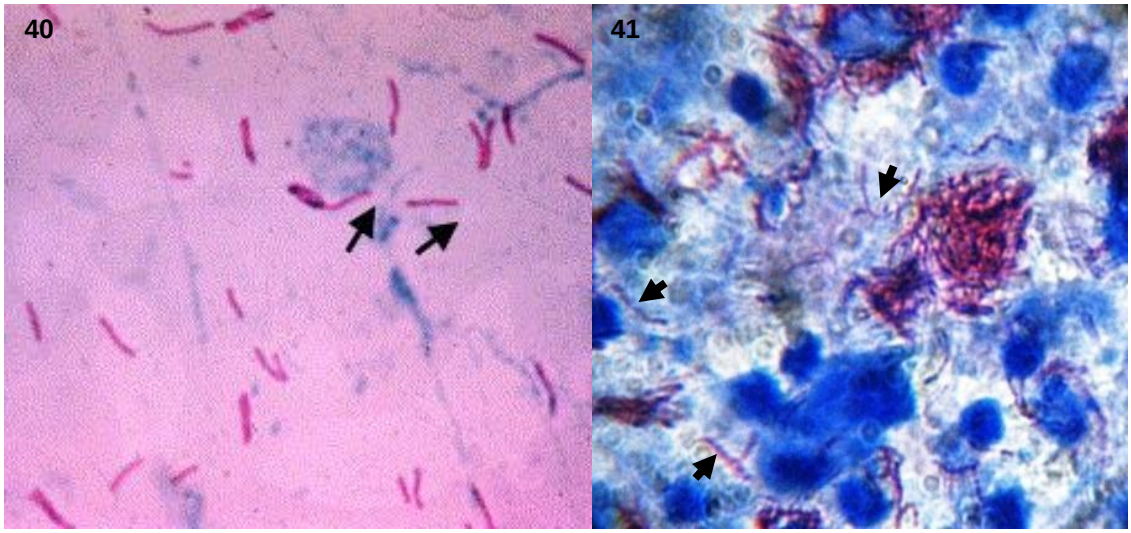
The threshold number of bacilli needed to obtain a positive microscopic exam (Ziehl Neelsen and other stains) is around 10^4 bacilli/mL, which is a rather important and infectious value (Lécu & Ball, 2011). Increased yields are obtained by immuno-fluorescent staining and detection of acid-fast bacilli (Mikota, 2008; Lécu & Ball, 2011). As the culture method, the amount of bacilli detected in biological samples may be affected by several factors, already mentioned above, for culture (Capuano et al., 2003; Miller, 2008; Lécu & Ball, 2014).

Acid-fast staining is one of the standard techniques used for TB testing in sloth bears. The most common, but not only, sample materials used are lung wash obtained through BAL, and a tracheal

smear, either obtained at *post-mortem* or at *ante-mortem in vivo* intubation when the animal in under anesthesia (Rishikesava et al., 2008; Renukaprasad et al., 2013; Arun et al., 2014).

Figure 40- Ziehl-Neelsen staining of *Mycobacterium tuberculosis* organisms (black arrows) in a human's sputum smear (Source: http://imnewbieschool.com/forum/language/mycobacterium-tuberculosis-acid-fast-bacilli-i15.jpg).

Figure 41- Ziehl-Neelsen staining of *Mycobacterium tuberculosis* organisms (black arrows) in a sloth bear's tracheal smear (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).



4.2.3. Molecular Methods

Methods for the diagnosis of tuberculosis have improved in recent years, and several molecular techniques for its diagnosis have been introduced for clinical use. Methods employing gene technology based on amplification and detection of bacterial DNA or RNA are expected to improve the speed, sensitivity, and specificity of mycobacterial detection (Dalovisio, 1996). These methods are able to identify nucleic acid from *Mycobacterium spp.* in clinical samples that are negative by microscopic examination, or determine whether acid-fast organisms, identified by microscopic examination, are *Mycobacterium spp.* or atypical mycobacteria (Abebe et al., 2007), showing the best sensitivity and specificity values when applied on pure culture material (Fitzgerald et al., 2000; Lécu & Ball, 2011). These methods can also identify the presence of genetic modifications known to be associated with resistance to anti-mycobacterial agents (Abebe et al., 2007).

4.2.3.1. Polymerase Chain Reaction (PCR)

PCR has been the most studied of the different molecular techniques that exist for species identification of *Mycobacterium* (Gamboa et al., 1997; Backues, 2008; Maas et al., 2013). It belongs to the category of Nucleic Acid Amplification Techniques (NAAT) and is widely used in all wildlife species for differentiation of mycobacteria of the *M. tuberculosis* complex from non-tuberculous mycobacteria, as well as for more specific differentiation among the members of the

M. tuberculosis complex, giving rapid results and having a greater sensitivity compared with traditional microbiological methods (Abebe et al., 2007; Lécu & Ball, 2011; Maas et al., 2013).
The reliability of PCR depends on the amplification of DNA with primers specific for different target sequences in the mycobacterial genome, and on optimal DNA isolation and PCR procedures (Deshpande et al., 2007). The repetitive nature of IS6110[21] insertion sequence in *M. tuberculosis* genome makes it an attractive target for PCR amplification, as it could contribute to a higher degree of sensitivity of the assay, being used in several studies (Dalovisio et al., 1996; Deshpande et al., 2007).
PCR techniques can be performed after culture or directly in the suspect samples (normally sputum and BAL but also possible to apply in feces) (Maas et al., 2013). It may also be performed on *post-mortem* samples, including formalin-fixed tissues (Backues, 2008; Miller, 2008), and to raw material (lavages, tissues), which decreases the delay before the results are available, although this application may compromise sensitivity and specificity as some of these biological samples are likely to host many other bacteria or biological compounds that are impairing PCR efficacy (Lécu & Ball, 2011).
This technique is widely used for *Mycobacterium* testing in sloth bears (Renukaprasad et al., 2013). It is usually coupled with culture and displays great sensitivity and specificity, especially at *post-mortem*, as samples are collected directly from tuberculous lesions (Mikota, 2008).

4.2.3.2 Amplified *Mycobacterium tuberculosis* Direct Test

The Amplified *M. tuberculosis* direct (AMTD)[22] test detects nucleic acid from the organism in clinical samples, providing rapid results (Mikota, 2008; Miller, 2008). It is also a NAAT and combines isothermal transcription-mediated amplification of a portion of the 16S rRNA with a detection method that uses a hybridization probe specific for *M. tuberculosis* complex bacteria, using respiratory sediments (sputum, tracheal aspirates and bronchial specimens), being highly sensitive and specific (Gamboa et al., 1997; Mikota, 2008). This technique has a rapid turnaround time (2.5-3.5 hours) and can detect low numbers of organisms (Mikota, 2008). In humans, the test is used in addition to acid fast bacillus (AFB) smear and culture (Dalovisio et al., 1996).
This test is designed for humans and it has not been applied in sloth bears yet.

4.2.3.3. Restriction Fragment Length Polymorphism (RFLP)

Also known as “DNA fingerprinting”, RFLP is used to identify and differentiate mycobacterial strains (Mikota, 2008). The technique involves cutting a particular region of DNA with known variability, with restriction enzymes, then separating the DNA fragments by agarose gel electrophoresis and determining the number of fragments and relative sizes (Phillips, 2016).

[21] IS6110 is a repetitive insertion sequence that is usually present 6-20 times in the M. tuberculosis genome (Deshpande et al., 2007).
[22] Gen Probe, San Diego, California, USA.

A restriction enzyme is an enzyme (protein molecule) that cuts DNA at restriction sites (specific recognition nucleotide sequences). In essence, the DNA sample is broken up and digested by the restriction enzymes. The resulting fragments are separated according to their lengths and the pattern of fragment sizes will differ for each individual tested (Phillips, 2016).
This technique is not applied in sloth bears, probably due to its complexity, its high cost[23] and the low available budget for diagnostic methods.

4.2.3.4. Spoligotyping

When there is sufficient DNA (rich sample or culture), molecular typing should be performed (Lécu & Ball, 2011). This method is based on polymerase chain reaction (PCR) amplification of a highly polymorphic direct repeat locus in the *Mycobacterium spp.* genome (Gori et al., 2005; Mikota, 2008). Spoligotyping allow researchers to identify strains of mycobacteria and determine the relatedness between them (Mikota, 2008; Lécu & Ball, 2011). Results can be obtained from a *M. tuberculosis* culture within 1 day. Thus, the clinical usefulness of spoligotyping is determined by its rapidity, both in detecting causative bacteria and in providing epidemiologic information on strain identities, with the possibility of being applied on raw samples (i.e. biopsies, fluids) (Gori et al., 2005; Lécu & Ball, 2014). Implementing such a method would be useful in surveillance of tuberculosis transmission and in interventions to prevent further spread of this disease (Gori et al., 2005).
Due to various reasons previously mention for other complex molecular diagnostic methods, this technique is not routinely used in sloth bears.

4.3. Indirect assays

Indirect tests detect antigens and antibodies or measure cellular reactivity against mycobacterial antigens (Mikota, 2008). The immune response to TB infection is both cell mediated and humoral (Mikota, 2008). In the early stages of tuberculosis, cell-mediated immune (CMI) responses predominate. High levels of antibody to mycobacteria usually occur in the advanced stages of the disease, when large numbers of organisms are present (de Lisle et al., 2002; Maas et al., 2013). The time frame of this shift from CMI response to humoral response, depends on many factors, namely host species and immunity, initial infection dose, re-infection, etc. The measurement of either or both stages of the immune response can be used for immunological diagnosis (Maas et al, 2013).

[23] Wildlife SOS is a non-governmental non-profit organization, financed exclusively by donations. When diagnosing TB in bears, naturally, only the most accessible and practical methods are selected.

4.3.1. Cell-Mediated Immunologic (CMI) Tests

During the CMI process, mycobacterial antigens are presented to naive T lymphocytes, which become activated, posteriorly producing various cytokines, the most prominent of all being Tumor Necrosis Factor (TNF) and Interferon-gamma (IFN-γ) (Chapel, Haeney, Misbah & Snowden, 2006; Lin et al., 2007; Ernst, 2012; Maas et al, 2013). Memory of T cells regarding previous contact with *Mycobacterium* can be assessed by presenting selected antigen(s) to them, either *in vivo* (Intradermal Skin Test) or *in vitro* (Lymphoblastic Transformation Test or Interferon-gamma tests) (Chapel et al., 2006; Maas et al, 2013).
CMI related diagnostic tests have the advantage of become positive in an early stage of the disease (Maas et al., 2013), and the fact that some can be performed over 24 hours (de Lisle et al., 2002; Montali et al., 2011).

4.3.1.1. *In vivo* Cell-Mediated Tests

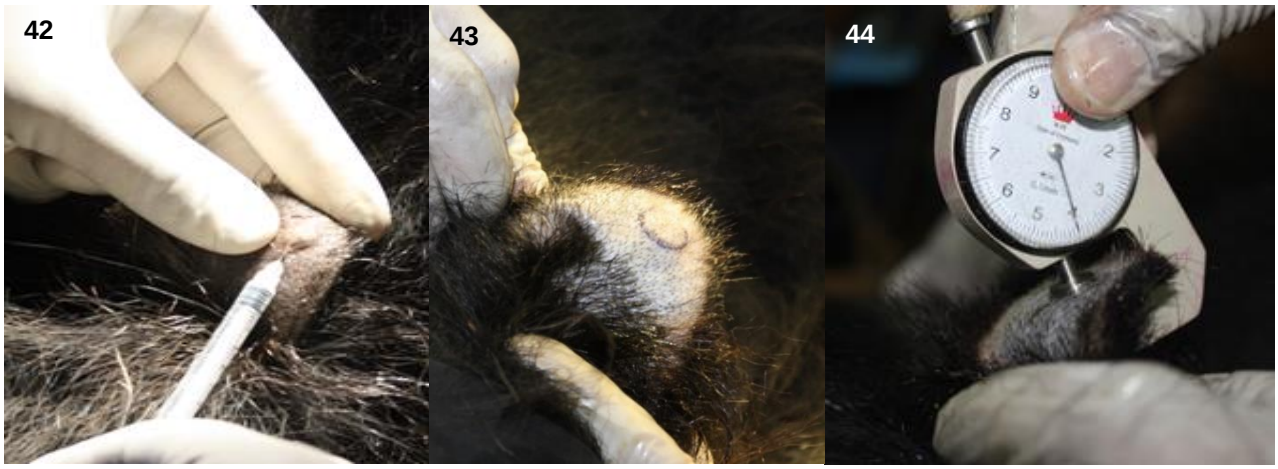
The intradermal tuberculin skin test (TST) is the most common *ante-mortem* TB diagnostic test used over the years in mammals, and it is based on *in vivo*, delayed-type hypersensitivity response to tuberculin antigens (Ciftci, Ilgazli, Gulleroglu, Ara & Akansel, 2005; Mikota, 2008; Miller, 2008; Lécu & Ball, 2014). However, several studies have shown the lack of value of this test for *M.tb* complex in carnivores (Backues, 2008).
While intradermal tuberculin testing had been attempted in a number of different wildlife species, it is far from being validated and standardized in these species (Mikota et al., 2000; Miller, 2008; Lécu & Ball, 2011; Maas et al., 2013) as it has some severe limitations and practical disadvantages: optimal tuberculin doses are often unknown; the re-recapture (which means two sedations) and examination of animals 48h to 96h (72h in sloth bears) after the injection of tuberculin is at the least impractical (de Lisle et al., 2002; Lécu & Ball, 2011); it can be affected by immunosuppressive status or superficial temperature (Lécu & Ball, 2011); exposure to environmental mycobacteria may cause high background values; and there are considerable variations between tegument and dermal structure within species for it to be used as a standard (Mikota et al., 2000; de Lisle et al., 2002; Maas et al., 2013).
In sloth bears, the test is performed on the ear lobe (Figures 42, 43 & 44), using 5 Tuberculin Units (TU) (= 0,1 ml) of Purified Protein Derivative (PPD) obtained from a human strain of *Mycobacterium tuberculosis* grown on a protein-free synthetic medium and inactivated (Renukaprasad et al., 2013). Although it has been tried, it is not currently used as a standard method, being a very unreliable test that correlates poorly with culture and *post-mortem*, and does not differentiate between a former infection and an active disease (Jurczynski et al., 2012; Arun et al., 2014). The reaction observed post-inoculation was not consistent within some positive

bears[24], being even present in some of the control animals, and the great thickness of this species skin plays a major role in the reaction evaluation (A.S. Arun, personal communication, April 20th, 2016).

Figure 42- Injection of PPD on a sloth bear's ear lobe (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Figure 43- TST reaction (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Figure 44- Measurement of TST reaction (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).



4.3.1.2. *In vitro* Cell-Mediated Tests

Cellular immunity *in vitro* tests rely on the (re)stimulation of T lymphocytes memory, and include lymphocyte transformation (LT), cytokine production (i.e., IFN-γ, interleukine-2) and other indirect measures of immunologic stimulation, such as cytokine ribonucleic acid (RNA) assays (Miller, 2008; Lécu & Ball, 2011).

In vitro cell-mediated tests for wildlife have the advantage of requiring only one handling of animals (Mikota et al., 2000; de Lisle et al., 2002). Besides this, they are *in vitro* tests, which removes some of the variability of *in vivo* tests, appearing to be more sensitive (Miller, 2008; Lécu & Ball, 2014). Also, cytokines are generally more conserved between species, so detection methods may be more widely applicable (Miller, 2008). Finally, these tests have a high importance for epidemiological and surveillance studies to determine the extent of TB infection in different populations as they allow an early detection of latently infected individuals, and are useful in contact-tracing and screening of high-risk groups in a low-endemic setting (Abebe et al., 2007).

The major limitation for *in vitro* tests of cell-mediated immunity is that blood samples have to be processed within hours after collection, which largely restricts the use of this test to research purposes (Mikota et al., 2000; de Lisle, 2002).

[24] Reaction considered positive with a measurement between 6mm and 8mm (A.S. Arun, personal communication, April 20th, 2016).

4.3.1.2.1. Lymphocyte Transformation Test

Lymphocyte transformation tests (LTT) are performed by stimulating mononuclear cells with specific antigens and then incubating the proliferating cells with a radioisotope-labeled nucleotide. The amount of label incorporated is related with the degree of proliferation, being an indicator of previous exposure and immune recognition of the specific antigen (Mikota et al., 2000; de Lisle 2002; Miller, 2008). Lymphocyte transformation assays do not require species-specific reagents and have been applied to a wide number of wildlife species, developed to overcome the problems associated with skin testing (Mikota et al., 2000; de Lisle 2002; Miller, 2008). LTT is not a current option for wildlife in general because of its limited reproducibility and the use of radio-elements (Miller, 2008; Lécu & Ball, 2011).

4.3.1.2.2. IFN-γ Release Assays

Interferon-gamma release assays (IGRAS) are now being used in veterinary medicine as an alternative to TST (Miller, 2008; Lécu & Ball, 2011; Fogel, 2015), although they are only validated for use in cattle, deer, non-human primates, and humans (Angkawanish et al, 2013). It studies and measures the *in vitro* cytokine (interferon-gamma) production by live lymphocytes contained in a fresh heparinized blood sample while exposed to selected mycobacterial antigens (Maas et al., 2013; Lécu & Ball, 2014). Interferon-γ release assays are highly effective for detecting latent infections but have the substantial limitation of requiring blood samples to be processed within 8-30 hours after collection, while white blood cells are still viable, which largely restricts the use of this test (Mazurek, Jereb, Vernon, LoBue, Goldberg & Castro, 2011; Lécu & Ball, 2014). Also, errors in collecting or transporting the blood specimens or in running and interpreting the assay can decrease the accuracy of IGRAS (Mazurek et al., 2011).

This test is performed in two steps, for a total run of 48h minimum. The first step is to incubate T cells with selected mycobacterial antigens, leading them to produce IFN-γ when they were previously in contact with the same antigen. The second step is to reveal the amount of IFN-γ produced through and ELISA or an ELISPOT (Figure 45) (EAZWV Tuberculosis Working Group, 2010).

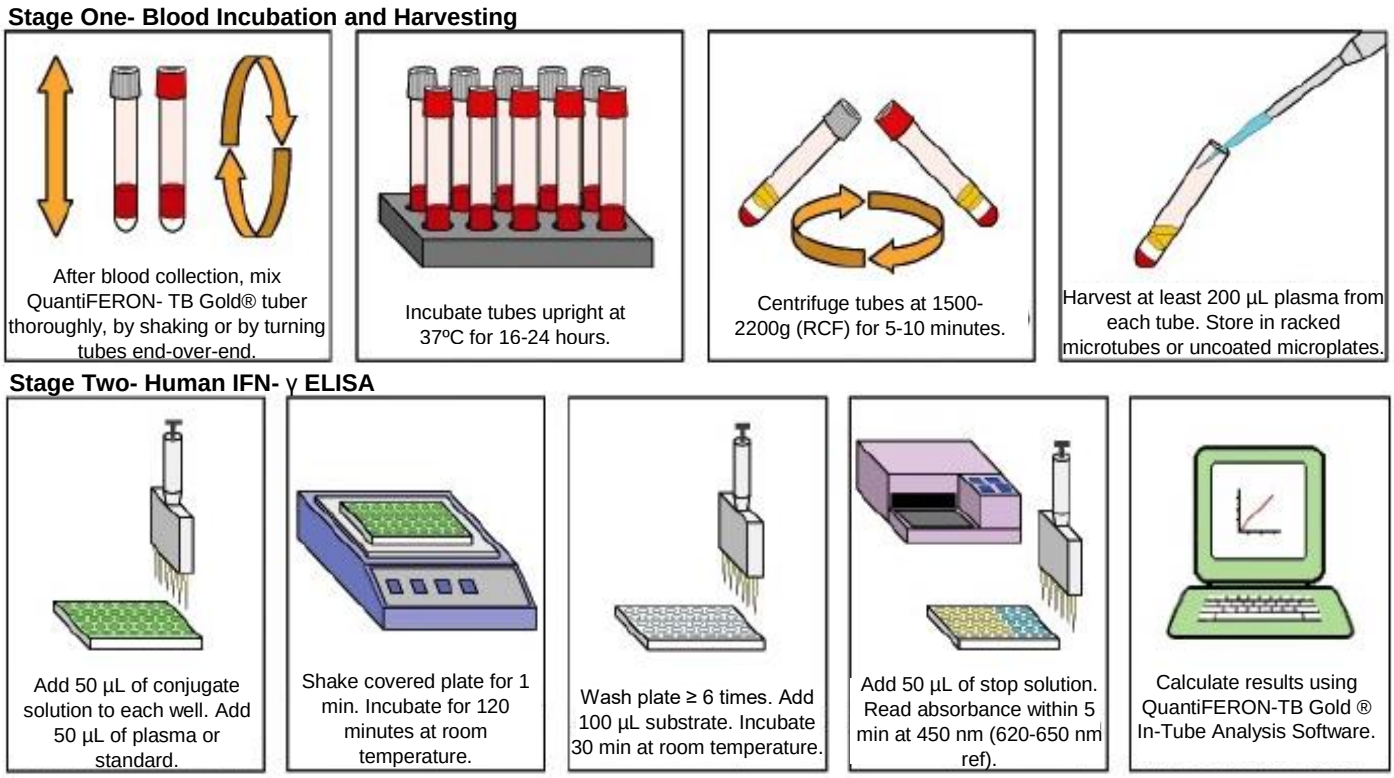
ELISA is used to detect the IFN-γ from a broader range of species (Maas et al., 2013; Lécu & Ball, 2014). This test was commercially available as PRIMAGAM®[25] for use in gorilla, orangutan, chimpanzee, gibbon, guereza, mandrill, squirrel monkey, marmoset, and baboon. A similar assay was produced for cattle (BOVIGAN®[26]), deer (CERVIGAN®[27]), and humans (QuantiFERON TB-Gold®[28]) (Miller, 2008; Lécu & Riquelme, 2008). Recent studies show that these tests can be used in some exotic species (Miller, 2008). In sloth bears, QuantiFERON TB-Gold® is the elected

[25] Prionics AG, Switzerland;
[26] Thermo Fisher Scientific, USA;
[27] Prionics AG, Switzerland;
[28] Cellestis Limited, Australia.

test for IFN-γ quantification (Arun et al., 2014), given its high availability in human medicine laboratories, to where most sloth bear samples are sent, and the correlation between bears' and humans' mycobacterial infection.

Resembling the skin test, the IFN-γ release assay has time limits because of decreasing CMI responsiveness during the progression of the disease (Maas et al., 2013).

Figure 45- Overview of the QuantiFERON-TB Gold® in tube assay technology (Source: http://www.bioline.org.br/showimage?mb/photo/mb05046f3.jpg).



4.3.1.2.3. Other assays

New researches investigating other cytokine production (e.g., IL-2) or cytokine RNA may provide additional *in vitro* methods of assessing response to mycobacterial infection (Miller, 2008). RNA sequences coding for IFN-γ primers are now available for a wide range of animal species, used to distinguish infected animals from non-infected animals (Lécu & Ball, 2014).

The methods previously mentioned are still very recent and none of them has been tried for TB screening in sloth bears.

4.3.2. Serological Tests

Simple diagnostic assays that are rapid, inexpensive and do not require highly trained personnel or a complex technological platform are essential to achieve global TB control (Abebe et al., 2007). Serology does not require a specimen from the site of disease and can be scaled up into a rapid, robust, inexpensive format requiring little laboratory infrastructure, having the many advantages of being simple, fast, relatively non-invasive and not requiring isolation or culturing of

the pathogen (Lyashchenko, Singh, Colangeli & Gennaro, 2000; Achkar, Lawn, Moosa, Wright, & Kasprowicz, 2011). It is therefore an especially attractive option for resource-limited settings and could ultimately serve as a point-of-care test (Achkar et al., 2011).

Recent tools, based on the investigation of humoral immunity, seem very promising for the detection of antibodies directed against mycobacterial antigens in a wide range of species (Miller, 2008), as many of them seem to present a strong, early, and persistent humoral component of their immune response to mycobacterial infections (Lécu & Ball, 2014).

There are different groups of T-helper lymphocytes (Th) that take part in the host's immune response against *M. tuberculosis*: Th1 participate in cell-mediated immunity by producing cytokines that activate other immune cells and Th2 provide help to B lymphocytes, which produce humoral responses (immunoglobulins) (Lécu & Ball, 2011; Ernst, 2012). During the course of an infection, Th1 activity (CMI pathway) is initially greater than Th2 (humoral route) in order to control and confine infection. An inversion of this Th1/Th2 balance is often associated with a relapse or with active disease (Lécu & Ball, 2011). It has also been noticed in humans (Abebe et al., 2007), cattle and wildlife, that some antibody rise occurs during the shift from latent to active disease, so that a prognostic value may be added to certain serological results (Lécu & Ball, 2011). As zoonotic risk increases a lot within this shifting time, detection of early serological change may be relevant to trigger a deeper screening (direct exam) and the implementation of preventive measures towards staff and public (Lécu & Ball, 2011).

Serum antibodies are produced against a variety of mycobacterial antigens and the number and type of serologically reactive antigens vary greatly among individuals (Lyashchenko, Colangeli, Houde, Al Jahdali, Menzies & Gennaro, 1998). This shows that a panel of mycobacterial antigens are able to elicit persistent humoral responses in a wide range of species (Miller, 2008; Lécu & Ball, 2011). The most relevant antigens have been selected to design tests like ELISA, MAPIA, and Rapid Lateral Flow Technology tests (Backues, 2008; Lyashchenko et al., 2008; Mikota, 2008; Greenwald et al., 2009).

4.3.2.1. Rapid Lateral Flow Technology Test

These assays are based on immune chromatographic/lateral-flow technology and some commercial tests, validated for Asian and African elephants (ElephantTB STAT-PAK®[29], Dual Path Platform [DPP] Vet TB®[30]), have showed promising results in non-target species (Backues, 2008; Lyashchenko et al., 2008; Greenwald et al., 2009), such as sloth bears, being widely used at Wildlife SOS for TB fast screening along with the Wild TB alert kit®[31] (Renukaprasad et al., 2013; Arun et al., 2014). The ElephantTB STAT-PAK® (Figures 46 & 48), the DPP VetTB® (Figure 47) and the Wild TB alert kit® (Figure 49) incorporate a unique cocktail of recombinant

[29] Chembio Diagnostics Systems Inc., NY, USA;
[30] Chembio Diagnostics Systems Inc., NY, USA;
[31] Genomix Biotech, India.

mycobacterial antigens impregnated on a nitrocellulose membrane (Lyashchenko et al., 2006; Lyashchenko et al., 2007, Mikota, 2008; Greenwald, 2009). Serum, plasma or whole blood may be used (Mikota, 2008). The ElephantTB STAT-PAK® and the Wild TB alert kit® require 20-30 µl of serum and 3 drops of sample buffer, and the DPP Vet TB® assay requires 5 µl of serum, 2 drops of buffer added to the sample well, and 4 to the conjugate well (Jurczynski et al., 2012). As the diluted sample migrates to the conjugate pad, the particles conjugated to the antigen bind to the antibody, if present in the sample, thus making the test band visible. In the absence of detectable antibodies, no specific immune complex would be formed on the test line, and therefore, no band would be visible. Results can be read 20 minutes after the addition of diluent (15 minutes for DPP VetTB®) (Lyashchenko et al., 2006; Lyashchenko et al., 2007; Lyashchenko et al., 2008; Mikota, 2008; Greenwald, 2009).

Figure 46- ElephantTB STAT-PAK® test function. (Source: Konstatin Lyashchenko, 2011, Elephant TB Seminar, in http://slideplayer.com/slide/6280770/).

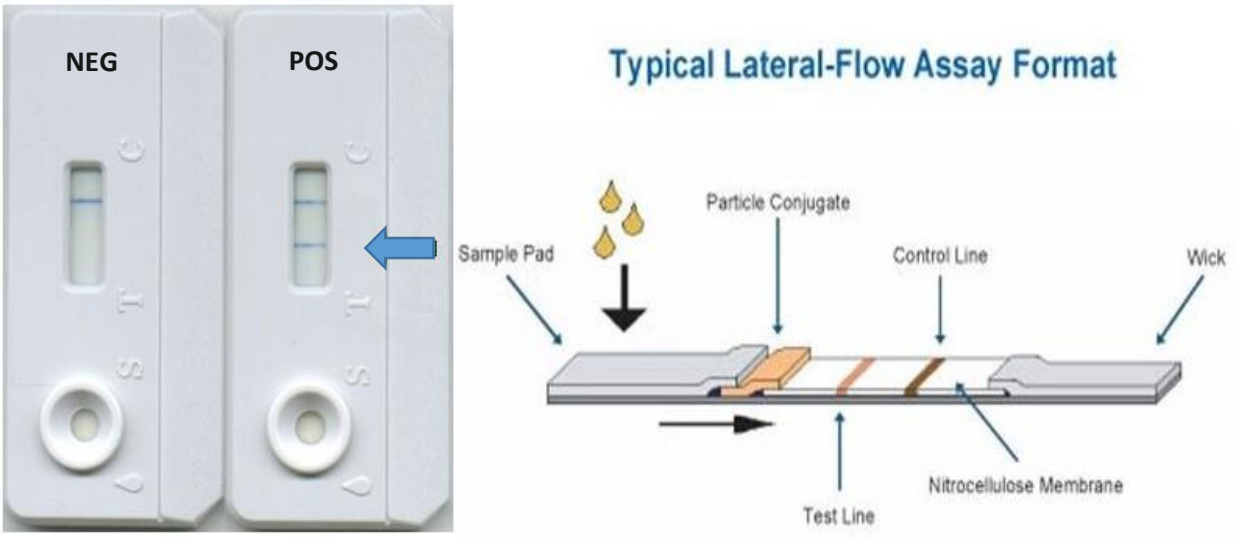
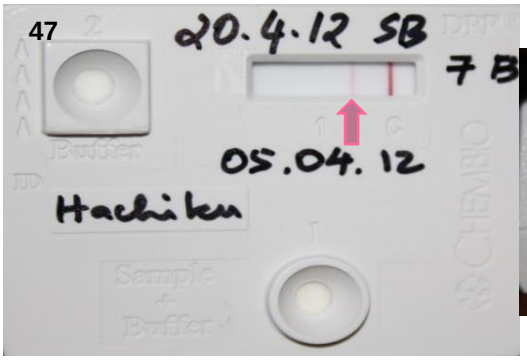


Figure 47- Positive DPP VetTB® test (pink arrow) from a sloth bear (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Figure 48- Positive ElephantTB STAT-PAK® (blue arrow) from a sloth bear (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).



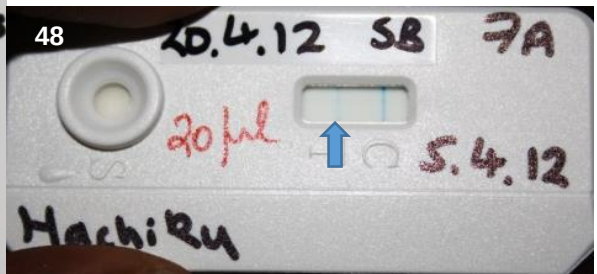
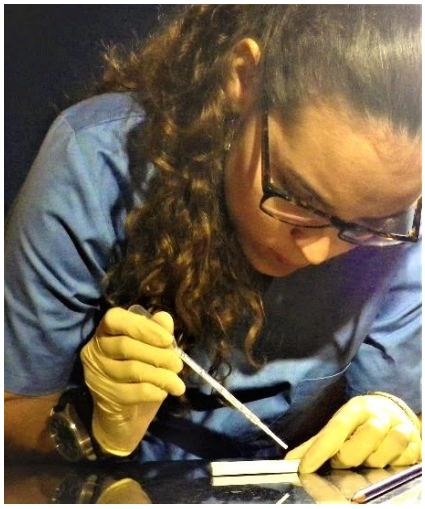


Figure 49- Rapid test execution (Wild TB alert kit®) using serum from a wild sloth bear blood sample. Serum should be added to the sample well located on the left side of the rapid test and the buffer after that (Original).



4.3.2.2. MAPIA and ELISA

The method called multi-antigen print immunoassay (MAPIA), is a laboratory procedure for antibody detection, based on the mobilization of antigens onto nitrocellulose membranes using an automated printing device (Lyashchenko et al., 2000; Mikota, 2008). Serum or a whole-blood sample are incubated with a MAPIA strip, and antigen-bound antibodies become visible using a species-specific immunoglobulin G (IgG)-binding enzyme conjugate and corresponding substrate (Mikota, 2008).

The enzymed-linked immunosorbent assay (ELISA) also measures antibodies against specific mycobacterial antigens and has been frequently used for laboratorial serologic TB diagnosis, especially and mostly for *M.bovis* detection in wild and domestic species. Nonetheless, it remains under investigation for wild species (Backues, 2008; Mikota, 2008; Miller, 2008).

With the use of rapid tests, which are cheap, fast and easy to perform, there has not been a need to resort to this technique in sloth bears.

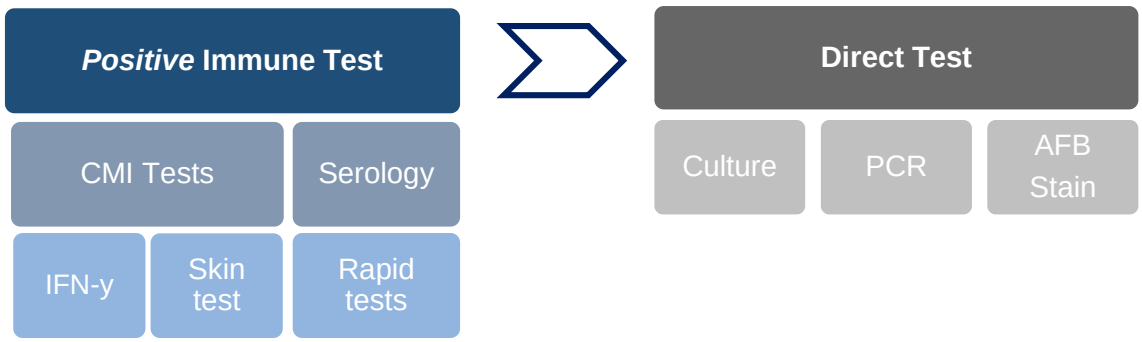
4.3.2.3. Other assays

Another example of an *ante-mortem* test, that is non-species specific and does not rely on host immune response, is antigen 85 (Ag85). Ag85 is a major secretory protein produced by actively growing mycobacteria (Backues, 2008). Antibodies to Ag85 of *M*. *tuberculosis* are produced and subsequently used to develop enzyme-linked immunosorbent assay (ELISA) for detecting Ag85 (Phunpae et al., 2014). This test has become an adjunct test in human medicine and has also shown promise in detecting *Mycobacterium* infections in other wildlife species, and, therefore, may be useful in the future in detecting infections in captive and free-ranging carnivores, as sloth bears (Backues, 2008).

4.4. Current screening recommendations

Even if available tools are still – and will likely remain – invalidated, crossing of the three exploration paths (i.e. direct, CMI and serological techniques) should be done, for each individual case. This way, the limitations of each type of test and the likelihood of false negatives and false positives are likely reduced (EAZWV Tuberculosis Working Group, 2010; Lécu & Ball, 2011). When we talk about wild species that live in close contact with humans, like in zoos or sanctuaries, the aim of detecting TB has two distinct levels. The first one is to protect the staff (keepers, vets) and, eventually, the public, from zoonotic contamination. Few actual tools are aimed at this predictive purpose, focusing on the active (i.e. excretion) phase of the disease. The second level is the census of latently infected individuals and their monitoring. For this purpose, CMI tests are used, as long as their sensibility/specificity limits are taken into account (EAZWV Tuberculosis Working Group, 2010; Lécu & Ball, 2011).
Any repeated positive outcome in the immunological tests should lead to exploration of the direct exam category (Figure 50), in order to determine the shedding status of the animal. If the direct exam shows a positive result, measures should be taken to avoid contamination of staff, surrounding animals and premises (EAZWV Tuberculosis Working Group, 2010; Lécu & Ball, 2011).

Figure 50- Positive immunological tests should trigger exploration of the direct exam category, schematic representation (Adapted from EAZWV Tuberculosis Working Group, 2010).



4.5. Sample Collection

4.5.1. *Ante-mortem*

Due to the asymptomatic nature of TB infection in sloth bears, an accurate and proper sample collection technique is critical in early diagnosis and subsequent treatment (Rishikesava et al., 2008; Renukaprasad et al., 2013; Arun et al., 2014). *Ante-mortem* diagnosis is the most difficult one to obtain but it also has a wider range of available biological samples to resort to (Montali et al., 2001). The animal must be sedated and all samples must be obtained in the most aseptic

conditions, especially the ones destined to culture and/or PCR (A.S. Arun, personal communication, April 20th, 2016).

Immune assays like IFN-γ assays and rapid tests, as well as blood analysis, require blood samples that are collected from the jugular at the time of the animal's sedation and general health examination. Samples for rapid tests are easily processed and fulfilled at BBRC (A.S. Arun, personal communication, April 20th, 2016).

Ante-mortem cytological techniques are usually used on lymph nodes (Martinho et al., 2013). Inguinal and subscapular lymph nodes are the ones more accessible to sample (A.S. Arun, personal communication, April 20th, 2016).

The various sample materials possible to collect and the *ante-mortem* diagnostics methods applicable for each case, are summarized in Table 8.

Table 8- Sample materials possible to collect *ante-mortem* and diagnostic methods applicable to each of them.

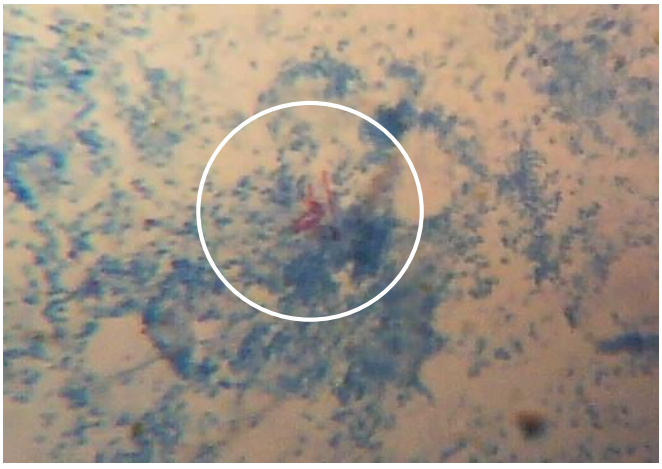
Material required	Diagnostic Test	How to obtain	Reference
Lung wash[32] (sputum)	AFB Staining, Culture, PCR	BAL **(Figures 51 & 52)**	**(Flynn et al., 2003; Arun, 2013; Arun et al., 2014)**
Tracheal swabs/ Smears (sputum)	AFB Staining, Culture, PCR	Intubation tube **(Figures 53 & 54)**	**(Montali et al., 2001; Arun, 2013; Arun et al., 2014)**
Nasal swabs	AFB Staining, Culture, PCR	Swab of nostrils **(Figure 55)**	**(Montali et al., 2001; Rishikesava et al., 2008; Arun, 2013)**
Oral swabs	AFB Staining, Culture, PCR	Swab of cheeks and oral cavity	**(Montali et al., 2001 Arun, 2013)**
Blood[33]	CBC, IGRAS, Serology	Blood collection	**(Arun, 2013; Arun et al., 2014)**
Serum	Serum chemistry, Serology	Blood collection	**(Arun, 2013; Arun et al., 2014)**
Urine	AFB Staining, Culture, PCR	US guided collection	**(Arun, 2013)**
Feces	AFB Staining, Culture, PCR	Rectal swab	**(Montali et al., 2001; Arun, 2013)**
Lymph nodes' aspirates	AFB Staining, Culture, PCR	Citology	**(Martinho et al., 2013)**

[32] Culture, PCR and IGRAS took place at the Dr. Lal Path Labs (human medicine laboratory), Bangalore until November 2015.
[33] Regular blood analysis, like serum biochemistry and complete blood count (CBC) took place at the Wild Animal Disease Diagnostic Laboratory, BBP Bangalore until November 2015.
[Since November 2015, all kinds of samples are taken to Oncquest Labs (human medicine laboratory) in Bangalore].

Figures 51 & 52- Sloth bear intubation for sputum collection through BAL (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Figures 53 & 54- After de-intubation it is possible to obtain a swab from the intubation tube for culture/PCR/smear or a direct smear for ZN staining from the exterior of the tube (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS)

Figure 55- Nasal sputum smear showing *Mycobacterium* spp. organisms (cylindrical structures stained pink) (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).



4.5.2. *Post-mortem*

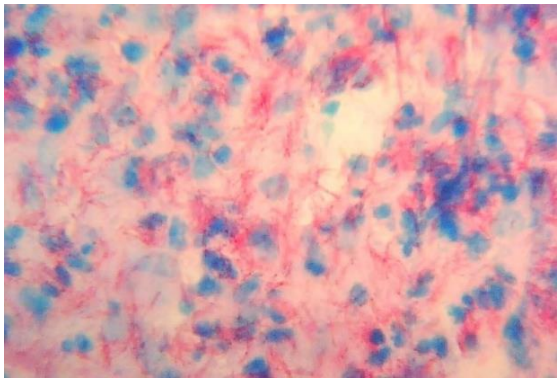
At *post-mortem*, it is possible to collect organ samples and search for the presence of mycobacteria organisms, using different techniques, providing a direct diagnosis. In most wildlife species, culturing, with possible subsequent PCR, has mainly been used as a *post-mortem* method, mostly using samples from affected lymph nodes and lung tissues (Maas et al., 2013; A.S. Arun, personal communication, April 20th, 2016).

In veterinary practice, lymph nodes from the respiratory tract, lung and liver tissue, lymph nodes from the gastrointestinal tract and pus or caseous material from open tuberculous cavities are, in that order, the specimens most frequently submitted for laboratory examination (Grange et al., 1996). It is mandatory that tissue samples collected during *post-mortem* examinations include normal and affected parenchyma (Renukaprasad et al., 2013; A.S. Arun, personal communication, April 20th, 2016).

As mentioned in the chapter "4.1.5. Histopathology", tissue samples for histology are normally formalin-fixed, embedded in paraffin wax, sectioned and stained with Hematoxylin-Eosin (HE) and Ziehl-Neelsen (ZN) (Fico et al., 2015). Smears for microscopic examination can also be collected during necropsy. The organ-impression smears can be taken from every affected organ (Figure 56), including lymph nodes, in aseptic conditions (Rishikesava et al., 2008; Renukaprasad et al., 2013).

Specimens should be placed in sterilized, wide-mouthed, hermetically sealable plastic or heavy glass containers and clearly labeled. When histological examinations are also required, the specimen should be divided in two and immediately put the portion designated for histology in 10% formalin. As laboratories are usually situated in urban areas (city of Bangalore, Karnataka state, in the case of BBRC) far from the park area, if transport is not immediately available, specimens should be kept cold by placing them in a +4ºC refrigerator, a container with ice or (if available) a freezer at -20 to -30ºC[34]. The latter prevents the growth of fungi (Grange et al., 1996).

Figure 56- Lung impression smear showing *Mycobacterium* organisms (cylindrical structures stained pink) (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).



[34] BBRC does not have electric power available, functioning with a generator that gathers energy from solar panels during the day, being only turned on when really needed (use of microscopes, use of operation theater in general, using of a small +4ºC refrigerator, etc.). This way, it is impossible to provide such temperatures during work hours, being only possible when the veterinary staff arrives to the Wildlife SOS house in Bannerghatta, at the end of the day. The samples stay in the house freezer until a sufficient number of samples are gathered, being then transported to the city of Bangalore, to the analysis laboratory.

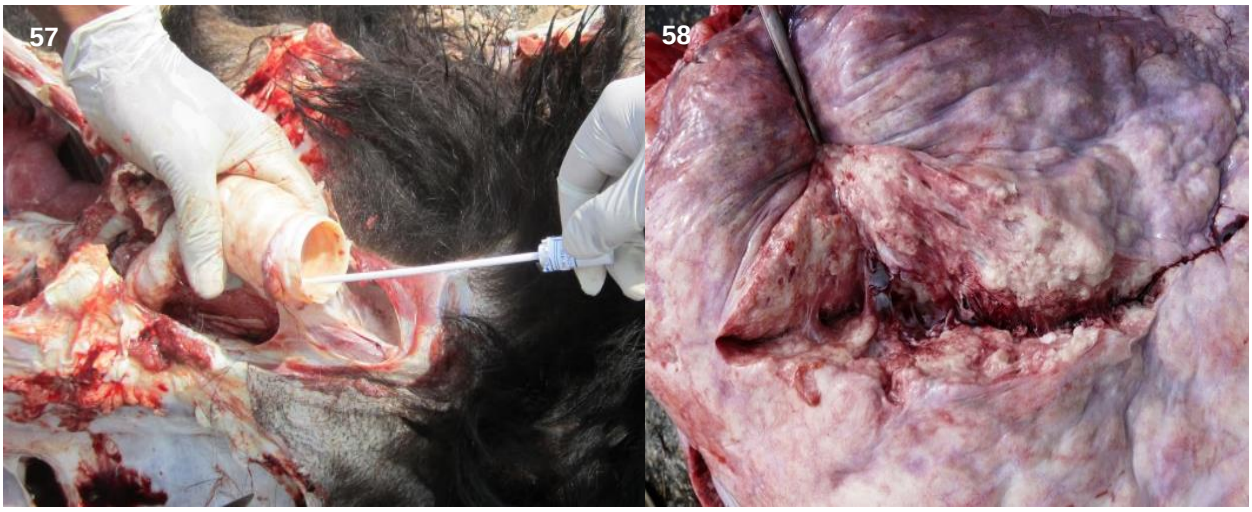
The various sample materials possible to collect and the *post-mortem* diagnostics methods applicable for each case, are summarized in Table 9.

Table 9- Sample materials possible to collect *post-mortem* and diagnostic methods applicable to each of them.

Material required	Diagnostic Test	How to obtain	Reference
Tracheal swabs/ smears	AFB Staining, Culture, PCR	Direct swab of the trachea at necropsy **(Figure 57)**	**(Montali et al., 2001; Arun, 2013; Arun et al., 2014)**
Nasal swabs	AFB Staining, Culture, PCR	Swab of nostrils	**(Montali et al., 2001; Arun, 2013)**
Oral swabs	AFB Staining, Culture, PCR	Swab of cheeks and oral cavity	**(Montali et al., 2001; Arun, 2013)**
Lymph nodes'/ Organs' sections	Histopathology, Culture, PCR, AFB Staining of organ impression smears	Biopsy **(Figure 58)**	**(de Lisle et al., 2002; Arun, 2013; Arun et al., 2014)**
Lymph nodes'/ Organs' aspirates	AFB Staining, Culture, PCR	Citology	**(Martinho et al., 2013)**
Feces	AFB Staining, Culture, PCR	Rectal swab	**(Montali et al., 2001; Arun, 2013)**

Figure 57- *Post-mortem* tracheal swab collection (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Figure 58- Lung section for sample collection (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).



5. Management Considerations for Positive or Suspected Animals

The choice of euthanasia or treatment should be considered carefully in concordance with veterinary staff, animal management, government officials and the respective studbooks of the species involved (Lécu & Ball, 2011; USAHA, 2012). A known infected animal should, normally, be subject of euthanasia and the premises disinfected with mycobacterial disinfectants (Backues, 2008). However, depending on TB extension analysis, treatment may be an alternative to euthanasia, to prevent infected animals from shedding mycobacteria. Treating an animal for TB implies following strict rules of drug administration, pharmacokinetic check, observance and excretion follow-up (Lyashchenko et al., 2006). A strict adherence to treatment schedules is required (Iallegio, 1997; A.S. Arun, personal communication, April 20th, 2016).

When considering treatment options, there must be awareness of the potential sequela resulting from a failure in treatment and the production of resistant strains of mycobacteria (Lyashchenko et al., 2006). The effect of a successful treatment is to bring the animal back to a latent stage, which means that reactivation will always be possible in any stage of its life, once the treatment is discontinued. The main point of treatment is rarely eradication. Treated animals should be monitored closely for the rest of their life (Lécu & Ball, 2011).

Euthanasia may be considered for those animals that are showing clinical signs, considered to be poor candidates for treatment. If euthanasia is elected, complete *post-mortem* examination should be conducted (Iallegio, 1997).

As sloth bears are a protected species in India, euthanasia is normally not authorized by the Government Forest Departments, even in the final stages of active disease, and treatment is the course of action elected. As a comparison, treatment is also applied in elephants (Mikota, 2008) but never in domestic dogs, given the high zoonotic risk (Paul, 2014).

5.1. Treatment

The guidelines for the treatment of TB in sloth bears, as there are no precedents for TB therapeutics in animals, are based on the assumption that animals with known active disease will respond to the treatment in a similar way as humans do (Thoen, 1993; Mikota, 2008; USAHA, 2012; A.S. Arun, personal communication, April 20th, 2016).

In the absence of diagnostic testing to confirm cure in sloth bears, the efficacy of these treatment regimens can only be understood as treated animals die and are examined *post-mortem* (USAHA, 2012; A.S. Arun, personal communication, April 20th, 2016). To this day, TB treatment in sloth bears only seems to succeed in the transition from an active to a latent stage, as affected (and treated) animals display marked lesions of tuberculosis at *post-mortem* examinations with detection of mycobacteria upon culture and PCR (A.S. Arun, personal communication, April 20th, 2016).

5.1.1. Treatment Protocols

As previously mentioned, the treatment protocol concentrates mostly in the use of human medication (USAHA, 2012; A.S. Arun, personal communication, April 20th, 2016). Anti-tuberculous agents are divided into first and second line agents. First line agents[35] used in the chemotherapy of TB include: isoniazid (INH), rifampin/rifampicin (RIF), pyrazinamide (PZA), and either ethambutol (EMB) or streptomycin (SM) (USAHA, 2012; Fogel, 2015). These are the agents with the greatest activity and the best side effect profiles. Second line agents include those with less activity and/or greater side effects (USAHA, 2012). As a thumb rule, no single anti-TB drug is used at the BBRC facility. It is also not advisable to combine both primary and secondary anti TB drugs (A.S. Arun, personal communication, April 20th, 2016).

INH and RIF are the elected ones for the TB treatment in sloth bears, being administered in syrup forms (R-cin®[36] and Iso-Caldin®[37]) in order to make the administration easier, by mixing the syrup with the porridge (each syrup in one of the two meals) (A.S. Arun, personal communication, April 20th, 2016). Both of them can also be aimed to treat active and latent tuberculosis infection, as the other three are only used in the treatment of active infection (National Institute for Health and Care Excellence [NICE], 2016).

For several years, the treatment period lasted from 5 to 6 months, continuously. If any bear skipped the treatment for one day, the course of administration would start over (A.S. Arun, personal communication, April 20th, 2016). Recently, a new treatment regime has been adopted by BBRC, which consists in the administration of INH and RIF every alternative day, for at least 6 months (A.S. Arun, personal communication, September 7th, 2016).

5.1.2. Dosages and Administration Routes

Anti-TB drugs vary in palatability and consequent acceptance, thus, some experimentation may be required to determine a workable regimen for each individual (USAHA, 2012; A.S. Arun, personal communication, April 20th, 2016). As an example, at BBRC, the start point was the administration of isoniazid and rifampicin as separate tablets, posteriorly a single tablet containing both of the substances, and, finally, a tablet containing 4 of the substances previously mentioned (isoniazid, rifampicin, pyrazinamide and ethambutol) (A.S. Arun, personal communication, April 20th, 2016). None of these was accepted by bears as they refused to ingest the food with it. Success was only totally achieved with the syrup formulations (A.S. Arun, personal communication, April 20th, 2016). The dosages and routes of administration for the main anti-TB drugs are described in Table 10.

[35] All the mentioned drugs are effective for both *M. tuberculosis* and *M. bovis* infection, with the exception of pyrazinamide that is not effective againt *M. bovis* (Mikota, 2008).
[36] Lupin Limited Pharmaceuticals, India;
[37] Retort Pharmaceuticals, India.

Table 10- Dosages and routes of administration of first line anti-TB drugs for treatment of tuberculosis infection in sloth bears at Wildlife SOS (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Drug	Dosage (mg/Kg)	Route
Isoniazid	**5**	**Oral**
Rifampicin	**10**	**Oral**
Pyrazinamide	25-30	Oral
Streptomycin	15	Injection (IM route)
Ethambutol	15	Oral

5.1.3. Side Effects

Animals should be closely checked for changes in appetite, attitude, and any other signs that may be attributable to adverse drug effects (USAHA, 2012; A.S. Arun, personal communication, April 20th, 2016).

The treatment previously described, using isoniazid as a first line anti-tuberculous drug, was shown to induce a pyridoxine deficiency (natural occurring form of vitamin B6) (A.S. Arun, personal communication, April 20th, 2016). Isoniazid interferes competitively with pyridoxine metabolism by inhibiting the formation of the active form of the vitamin, which is involved in many metabolic processes, like hemoglobin synthesis, amino acid metabolism and neurotransmitter synthesis (Snider, 1980; Combs, 2012). Hence, this treatment often results in peripheral neuropathy, muscular weakness and anemia (Snider, 1980; Combs, 2012; A.S. Arun, personal communication, April 20th, 2016). Hepatotoxicity is also frequently reported as an adverse reaction during the treatment with both isoniazid and rifampicin (Houston & Fanning, 1994). This way, sloth bears can be seen staggering, falling and tripping while walking, and with high levels of liver enzymes upon blood analysis (A.S. Arun, personal communication, April 20th, 2016).

Investigations suggested that concurrent administration of pyridoxine would protect against the development of INH peripheral neuropathy in humans (Snider, 1980). This way, B-complex supplements are administered prophylactically to sloth bears along with the anti-TB medication[38] (A.S. Arun, personal communication, April 20th, 2016).

Periodic blood tests (Complete Blood Count [CBC] and serum chemistry profile) are recommended to monitor general health and possible drug effects on the liver (Mikota, 2008; USAHA, 2012).

[38] Though Iso-Caldin® syrup already contains pyridoxine [(isoniazid150 mg + piyridoxine 1.5 mg) / 5ml] in its composition, supplementation is still required.

5.1.4. Mechanism of Action

5.1.4.1. Rifampicin

Rifampicin is an essential element of modern short course (Houston & Fanning, 1994). It inhibits the activity of bacterial DNA-dependent RNA polymerase, thereby inhibiting synthesis of mRNA and new protein chains (USAHA, 2012; Grange, 2014).

5.1.4.2. Isoniazid

Isoniazid has potent bactericidal activity against all populations of organisms, by inhibiting the biosynthesis of mycolic acids, the major components of *M. tuberculosis* cell wall (Houston & Fanning, 1994).

5.1.4.3. Pyrazinamide

Pyrazinamide has a potent sterilizing action in the acid pH of the intracellular environment (Houston & Fanning, 1994). The exact mechanism of action by which pyrazinamide inhibits the growth of *M. tuberculosis* organisms is unknown, but there is evidence that it disrupts synthesis of adenosine triphosphate (ATP), essential for energy generating processes within the bacterial cell (Grange, 2014).

5.1.4.4. Ethambutol

Ethambutol diffuses into actively growing *M. tuberculosis* such as tubercle bacilli. Ethambutol appears to inhibit the synthesis of the major cell wall polysaccharide arabinogalactan, causing impairment of cell metabolism, arrest of multiplication and cell death (Grange, 2014).

5.1.4.5. Streptomycin

Streptomycin is an aminoglycoside antibiotic that acts on the 30S[39] ribosome to inhibit protein synthesis (USAHA, 2012).

6. Prophylaxis

There are some features of infections with *M. tuberculosis* that allow the possibility of chemoprophylaxis: the long interval between infection and active symptomatic disease; the fact that the bacterial population during this interval is small (usually < 10^5 organisms); and the fact that the frequency of naturally occurring isoniazid resistance is very low in the mycobacterial population (USAHA, 2012).

[39] Smaller subunit of the 70S ribosome, found in prokaryote organisms.

The decision of implementing isoniazid and rifampicin chemoprophylaxis is based on the likelihood of infection (high risk of exposure), the risk of progression to active disease and the risk of hepatotoxicity (USAHA, 2012; A.S. Arun, personal communication, April 20th, 2016).
All new arrived animals at BBRC, as previously mentioned, are submitted to several exams and TB screening. Even if the results come back as negative for tuberculosis, a prophylactic course of treatment will be applied (A.S. Arun, personal communication, April 20th, 2016).

6.1. Drug Protocols

The dosages for isoniazid and rifampicin in prophylactic protocols are the same as the ones mentioned in Table 10, but the period of administration will last only for 60-90 days (A.S. Arun, personal communication, April 20th, 2016).

6.2. Vaccination

Vaccination is a key strategy for tuberculosis control and considerable progress has been made to develop improved vaccines for humans, domestic animals and wildlife (Buddle, Parlane, Wedlock & Heiser, 2013).
The BCG vaccine (Bacillus Calmette-Guérin), used in humans and cattle, has already been applied in some wildlife species like possums, badgers, white-tailed deer, wild boar and African buffalo, although it was shown in several studies that the efficacy level of protection varies between species. The vaccine can be administered orally or by injection, either by IM or SC route (Buddle et al., 2013).
Vaccination is not applied, neither was ever experimented on sloth bears, despite the BBRC veterinary staff's efforts to implement a protocol regarding it (A.S. Arun, personal communication, April 20th, 2016).

6.3. Other Preventive Measures

All newly arrived animals should be submitted to a meticulous general health examination, blood and/or sputum collection for TB screening, and to a period of quarantine (90 days) in order to guarantee the safety of all animals and staff (Rishikesava et al., 2008; A.S. Arun, personal communication, April 20th, 2016).
Providing a positive environment, balanced nutrition, supplements, routine deworming drugs, social well-being, and avoid stress, is essential to keep animals in good condition and with a functional immune system (Mikota, 2008; USAHA, 2012; A.S. Arun, personal communication, April 20th, 2016).
Providing a hygienic environment with little contamination is also an important step towards a healthy and safe living area (USAHA, 2012). This is currently acquired by the use of foot-dips with Potassium permanganate ($KMnO_4$) (Figure 59) at the entry of each bear enclosure at BBRC, and

a daily cleaning and disinfection of all the enclosures (A.S. Arun, personal communication, April 20th, 2016).

Continuous observation of every animal kept within the sanctuary is mandatory as clinical signs of actively sick bears are unspecific and appear at a very late stage of infection, making it necessary to act rapidly and effectively. It is also relevant to perform routine sedations with diagnostic testing in order to identify positive bears and isolate them from others (A.S. Arun, personal communication, April 20th, 2016).

The staff working in close contact with the bears must take adequate preventive hygiene measures, by using Potassium permanganate ($KMnO_4$) footbaths, masks and gloves (Figure 60) to avoid cross contamination (Davis, 2001; Mikota, 2008; Rishikesava et al., 2008; A.S. Arun, personal communication, April 20th, 2016). Also, every 6 months, the staff that works in direct contact with the animals should go through a routine TB screening (TST every 6 months and thoracic X-rays every 12 months), and new employees should be tested prior to contact with the animals (Davis, 2001; Backues, 2008; Mikota, 2008; Rishikesava et al., 2008; USAHA, 2012; A.S. Arun, personal communication, April 20th, 2016).

It is essential that a *post-mortem* examination is performed on all deceased animals. The examination must include a thorough search for tuberculosis lesions and posterior culture, regardless of exposure status (Davis, 2001; USAHA, 2012).

Adequate care to ensure safe disposal of all biological waste, should be provide (Davis, 2001; USAHA, 2012; A.S. Arun, personal communication, April 20th, 2016).

Figure 59- Potassium permanganate footbaths, located at the entrance of every bear enclosure (Courtesy of Dr. Susan Varghese M., Bannerghatta Bear Rescue Centre, Wildlife SOS).

Figure 60- When dealing closely with bears or biological materials, the use of gloves, masks and caps is imperative (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

7. Differential Diagnosis

Some diseases may be taken in consideration when dealing with a suspicion of tuberculosis infection in sloth bears. The previous symptoms presented as typical for tuberculosis are, in fact, common for a variety of diseases.

Since the primary clinical signs are based on weight loss, anorexia, anemia and digestive symptoms, conditions as parasitism, mal-nutrition, dental and liver disorders should be considered as possible diagnosis (Mikota, 2008; A.S. Arun, personal communication, April 20th, 2016). Also, pulmonary lesions caused by other organisms may be macroscopically mistaken by lesions caused by mycobacteria (Begins, 1999; de Lisle et al., 2002; Mikota, 2008) either at *post-mortem* (de Lisle et al., 2002) or on the X-ray examination. These differential diagnoses include actinomycosis, actinobacillosis, nocardiosis, pseudotuberculosis, histoplasmosis, cryptococcosis, and blastomycosis, to name a few (Beggins, 1999).

Degenerative diseases affecting the major body organs may also present with similar signs as TB (Mikota, 2008).

Chapter IV- Study

1. Aims of the Study

The primary aim of this study was to estimate the sensitivity of the different tests that are mostly used to diagnose tuberculosis infection in sloth bears that live in captivity in rescue centres, more accurately, at Wildlife SOS, Bannerghatta Bear Rescue Centre, in Karnataka, India.
Since there is no test specifically designed for bears, it is of great importance to designate which ones have the highest sensitivity, in order to achieve a diagnosis as reliable as possible. This matter carries an even higher importance and urgency when we account the fact that we are dealing with a very emblematic zoonosis.
As stated in previous chapters, when there is no validated or specific diagnosis for certain species, as it is the case, crossing of all specific exploration paths (i.e. direct, CMI and serological techniques) should be done, in order to reduce the limitations of each type of test and the likelihood of false negatives and false positives (EAZWV Tuberculosis Working Group, 2010; Lécu & Ball, 2011), which will naturally increase the chances of a successful *ante-mortem* diagnosis. Therefore, it was also an intent of this study to infer the combination(s) of *ante-mortem* tests that would suit this purpose the best.

2. Material and Methods

2.1. Inclusion Criteria

2.1.1. Specimen Information

In this analysis, only previous "dancing (sloth) bears"[40] rescued by Wildlife SOS and housed at BBRC were included. The 15 animals included in this study were the ones that, on BBRC records, (1) were considered positive upon their arrival at the centre; (2) had the highest number of different types of *ante-mortem* tests applied on them in the years that followed (with several positive results), allowing the collection of the highest possible number of cases for analysis[41].
At BBRC, sloth bears are routinely sedated for examination (more or less every 12 months) and the tests in question could have been taken at any time in their lives and not necessarily in the same moment[42].
Fourteen of these 15 bears died, between 2008 and 2013, and *post-mortem* tests were applied on the same as well. All 14 cases were confirmed positive for TB at *post-mortem*. All the presented

[40] BBRC houses mostly bears rescued from a "dancing" past with the Kalandar community, but not only. "Conflict animals", threatened by human populations, and wounded ones, also have a place at the Bannerghatta Bear Rescue Centre.
[41] It is important to mention that all the information regarding animals and previous testing is registered only in paper files, not particularly easy to find, analyze and correlate.
[42] The exception to this is PCR and culture, which are done at the same time and using the same sample material.

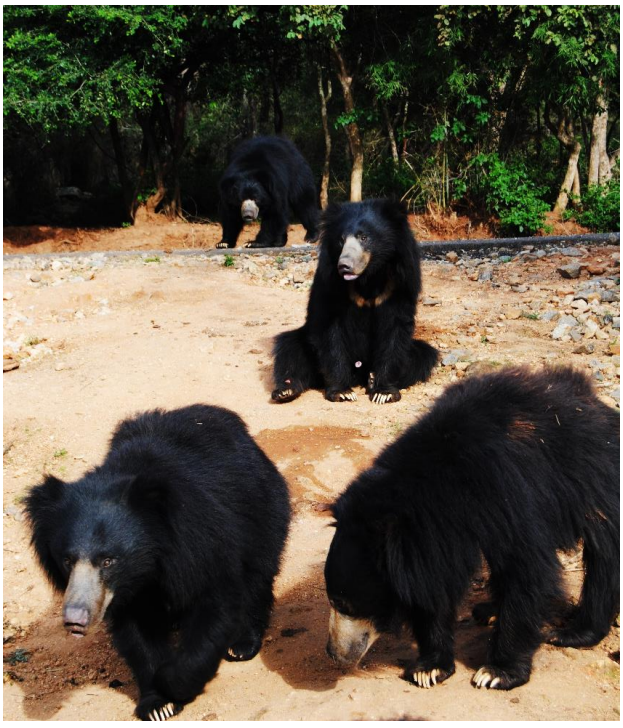
data was collected by Dr. A. Sha. Arun and the rest of BBRC veterinary team between 2006 and 2014, as this was the last date that a test was applied on the live animal.

Table 11- Specimen information (Courtesy of Dr. A. Sha. Arun and Dr. Susan Varghese M., Bannerghatta Bear Rescue Centre, Wildlife SOS).

Animal	Gender	Age (y)	Year of death
Bear 1	F	19	NA
Bear 2	F	18	2012
Bear 3	M	17	2013
Bear 4	M	14	2012
Bear 5	M	12	2012
Bear 6	M	15	2012
Bear 7	M	15	2011
Bear 8	F	16	2011
Bear 9	M	13	2012
Bear 10	M	17	2011
Bear 11	M	23	2008
Bear 12	F	14	2011
Bear 13	M	11	2011
Bear 14	F	15	2011
Bear 15	M	11	2013

NA- Not available.

Figure 61- All animals in this study were rescued “dancing bears” that were hosted at Bannerghatta Bear Rescue Centre, Wildlife SOS (Original).



2.1.2. Testing Considerations

Although numerous tests are described in the chapter “4. Diagnosis” of this dissertation, only the ones routinely used at BBRC will be subject of study. In order to provide the reader a better understanding of the conditions each method took place in, all the information regarding each diagnostic test will be addressed next.

Clinical Signs: As animals roam around constantly, it is not simple to evaluate their health status, especially as TB related symptoms are usually absent or nonspecific despite extensive tissue involvement, especially of the lungs. None the less, as anorexia is one of the most frequently observed signs, animals are closely monitored during feeding time, which happens twice a day (11am and 4pm). If any bear skips a meal, it is registered on the “feeding records file” and if that behavior keeps being repeated, the bear will be kept under closer observation and care. Other symptoms as vomiting, weakness, dullness, cough and respiratory distress will be registered once they manifest themselves and the veterinary team will decide the best course of action to follow.

Chest Radiography: Different imaging techniques can also play a role in TB diagnostics. Chest radiography is the only method of imaging used in sloth bears to detect tuberculous lesions. Lesions are more easily detected when they are calcified, which does not always occur. The sloth bears of this study were first sedated with an IM route injection of xylazine (2 mg/kg) and ketamine (5 mg/kg) and transported to the BBP Zoo[43], where the proper material to perform a body X-rays was set.

Clinical Pathology: Blood samples were collected whenever the animals were chemically sedated and transferred to EDTA (in order to obtain whole blood for CBC) and plain tubes (in order to obtain serum for biochemistry). The samples were then kept in +4 degrees Celsius and transported to the Wild Animal Disease Diagnostic Laboratory, in Bannerghatta.

Gross Pathology: Necropsy examinations were performed at BBRC to 14 of the 15 bears in this group. Samples of lesions and non-lesional tissues (mainly lymph nodes and lungs) were collected and shipped to an exterior human medicine laboratory, Dr. Lal Path Labs, in Bangalore, where culture and PCR were performed.

Culture: The materials potentially containing the organism were cultured in Löwenstein-Jensen medium. Whenever there was a positive result, colonies with a typical buffy appearance were revealed. The sample material used was sputum (tracheal swab/lung wash) in *ante-mortem* screenings, and materials collected from tuberculous lung lesions and lymph nodes (thoracic and

[43] BBRC does not own a full body X-ray, only a dental X-ray. In order to get a chest radiography, the animal, accompanied by the veterinary staff, is dislocated to the BBP Zoological Garden Veterinary Hospital facilities.

mesenteric) in *post-mortem* examinations. It usually takes between 6-12 weeks (average of 8 weeks) for results to appear.

PCR: This nuclear acid amplification technique was performed using the same sample materials as the ones mentioned for culture.

Acid-fast Bacilli (AFB) Staining: The test was carried out using sputum from trachea smears, obtained by intubation, stained by the Ziehl-Neelsen (ZN) method and analyzed microscopically under oil immersion lens. This test was performed at BBRC and only as an *ante-mortem* method, though it could, and should, be performed *post-mortem*.

Tuberculin Skin Testing: The test was performed on the ear lobe, using 5 Tuberculin Units (TU) (0,1 ml) of Purified Protein Derivative (PPD) of *M. tuberculosis*. The skin reaction was evaluated 72h later, requiring two separate sedation moments. All interventions were performed at BBRC.

Interferon-gamma Release Assays (IGRAS): Using whole blood samples, the QuantiFERON-TB Gold® for human testing is the test of choice for IFN-γ detection in sloth bears. This test was performed in an exterior human medicine laboratory, Dr. Lal Path Labs, in Bangalore.

Serology (Rapid Tests): The three rapid tests (Wild TB alert kit®, ElephantTB STAT PAK® and DPP VetTB®) can be performed by using either whole blood, serum or plasma. In this study only serum was used for all three of them. All tests were performed at BBRC, after centrifugation of the whole blood samples, soon after collection.

2.1.3. Treatment Considerations

Treatment combining rifampicin and isoniazid had been carried out at some point in these animals' lives, after the occurrence of positive results upon their arrival at the centre and consequent general health examination and TB screening.
It needs to be kept in mind that treatment of exposed animals, if initiated early enough, may prevent the clinical manifestation of infection and there is also a strong indication that treatment might succeed in eliminating the bacteria in animals (Jurczynski, 2012), as it does in humans.
In sloth bears, the major achievement registered was a transition to the latent stage (A.S. Arun, personal communication, April 20th, 2016).

2.2. Data Analysis

In this study, tests were divided, first by the moment they took place (*ante-mortem* or *post-mortem*) and then, for the *ante-mortem* tests, by the three major existing categories (unspecific, direct and indirect tests).

At the beginning of this study, the 15 animals in question were considered TB positive when, through their living years, several different types of tests were indicative of a positive TB status. However, the *post-mortem* tests were considered the ultimate confirmation and, consequently, the gold-standard methodology in this case (although it was only viable to apply them on 14 bears).

This way, taking the assumption above, there were no negative animals within this study group, which means that it was not possible to calculate the specificity (true negatives among all the negative results) of each test in question, only the sensitivity (true positives among all the positive results). With this said, the sensitivity was calculated for each and every method using the following formula:

$$\text{Sensitivity (\%)}^{44} = \frac{\text{TP}^{45}}{(\text{TP} + \text{FN}^{46})} \times 100$$

Once the sensitivity was obtained, the most suitable tests were combined in sets of two and the sensitivity re-calculated using a parallel testing approach, meaning that if at least one of the two tests in question was positive for each bear, the animal would be considered positive.

The same was made for a combination of three tests belonging to the major three exploration paths (direct, CMI and serological), considering positives the bears with either two positive tests out of three and one positive test out of three.

The sensitivity values for every test and test combinations were also graphically compared. The graphics were created using Microsoft Excel 2010®.

A 95% confidence interval (CI) was calculated for every test sensitivity obtained, with the R® program, version 3.3.1, using the extension R Studio® (The R Foundation for Statistical Computing, 2016).

Although it is not a matter in this quest, it is important to keep in mind that the type of approach applied in the combination phase, while increasing the sensitivity, will also decrease the specificity.

[44] The results were presented in percentage (%) and rounded to one decimal place;
[45] True Positive;
[46] False Negative.

3. Results

3.1. *Ante-mortem* Tests

3.1.1. Unspecific Tests

The results of the unspecific *ante-mortem* tests for every analyzed animal, calculated sensitivity and its confidence interval are presented next, in Table 12.

Table 12- Results for the unspecific *ante-mortem* tests applied to all 15 bears and calculated sensitivity (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Test	Early Clinical Signs	Chest X-ray	Biochemistry	Hematology/CBC
Sample material	-	-	Serum	Whole blood
Bear 1	N	ND	N	N
Bear 2	N	N	N	N
Bear 3	N	N	N	N
Bear 4	N	N	N	N
Bear 5	N	N	N	N
Bear 6	N	ND	N	N
Bear 7	N	N	N	N
Bear 8	N	N	N	N
Bear 9	N	N	N	N
Bear 10	N	P	N	N
Bear 11	N	P	N	N
Bear 12	N	N	N	N
Bear 13	N	N	N	N
Bear 14	N	N	N	N
Bear 15	N	N	N	N
Total	**0/15**	**2/13**	**0/15**	**0/15**
Sensitivity (%)	**0**	**15.4**	**0**	**0**
Confidence Interval (%)	**0-30**	**2-45**	**0-30**	**0-30**

ND- Not Done; P- Positive; N- Negative

None of the animals showed any kind of early clinical signs, like gastrointestinal symptoms, weakness, anorexia, dullness, etc., being asymptomatic until the disease was well advanced or not showing signs at all.

Out of the 13 bears submitted to X-ray examinations, only 2 showed radiographic signs that match the image of tuberculous lesions, displaying a sensitivity of 15.4%.

Laboratory analysis of the bears' blood showed no conclusive features of tuberculosis infection, even considering the natural lack of specific hematological and biochemical features.

3.1.2. Direct Tests

The results of the specific direct *ante-mortem* tests for every analyzed animal, calculated sensitivity and its confidence interval are presented next, in Table 13.

Table 13- Results for the direct *ante-mortem* tests applied to all 15 bears and calculated sensitivity (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Test	**Culture**	**PCR**	**AFB- ZN Staining**
Sample material	Lung wash/ Tracheal swab		Lung wash/ Tracheal smear
Bear 1	N	N	N
Bear 2	N	N	N
Bear 3	N	N	N
Bear 4	N	N	P
Bear 5	N	N	P
Bear 6	N	N	P
Bear 7	N	N	N
Bear 8	N	N	P
Bear 9	N	N	N
Bear 10	N	N	N
Bear 11	N	N	N
Bear 12	N	N	N
Bear 13	N	N	N
Bear 14	N	N	N
Bear 15	N	N	P
Total	**0/15**	**0/15**	**5/15**
Sensitivity (%)	**0**	**0**	**33.3**
Confidence Interval (%)	**0-30**	**0-30**	**12-62**

P- Positive; N- Negative

Using material (sputum) obtain with a Broncho-Alveolar Lavage (BAL) and tracheal swabs, all culture mediums came back negative, as well as the nucleic acid amplification (PCR) technique. On the other hand, the Acid-fast staining technique (Ziehl Neelsen method), was able to detect the organism present in the tracheal smears in 33.3% of the cases.

3.1.3. Indirect Tests

The results of the specific indirect *ante-mortem* tests for every analyzed animal, calculated sensitivity and its confidence interval are presented next, in Table 14.

Table 14- Results for the indirect *ante-mortem* tests applied to all 15 bears and calculated sensitivity (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Test	**CMI Tests**		**Serological Tests**		
	TST	QuantiFERON-TB Gold®	ElephantTB STAT-PAK®	DPP VetTB®	Wild TB alert kit®
Sample material	-	Whole Blood	Serum	Serum	Serum
Bear 1	ND	P	P	N	P
Bear 2	P	P	P	ND	N
Bear 3	ND	P	N	N	P
Bear 4	P	N	P	N	P
Bear 5	P	P	N	N	P
Bear 6	P	N	N	ND	P
Bear 7	P	N	P	P	P
Bear 8	ND	P	P	P	N
Bear 9	ND	N	N	P	P
Bear 10	P	P	P	N	P
Bear 11	P	P	P	N	P
Bear 12	P	N	P	P	P
Bear 13	P	N	N	N	N
Bear 14	ND	N	P	P	P
Bear 15	ND	P	N	N	P
Total	**9/9**	**8/15**	**9/15**	**5/13**	**12/15**
Sensitivity (%)	**100**	**53.3**	**60**	**38.5**	**80**
Confidence Interval (%)	**55-100**	**27-79**	**32-84**	**14-68**	**52-96**

ND- Not Done; P- Positive; N- Negative

It was not possible to apply the Tuberculin Skin Test in 40% of the group, but it showed positive results in all 9 animals inoculated, with reactions that were considered undoubtedly positive after evaluation, with an assumption of 100% of sensitivity.

Using the QuantiFERON-TB Gold®, we were able to obtain a 53.3% sensitivity, as 8 bears among the 15 showed positive results. As for the three rapid immune assays, the Wild TB alert kit® was able to reveal positivity for 80% of animals, followed by the ElephantTB STAT-PAK® with 60% and, finally, the DPP VetTB® with only 38.5% of positive outcome, although it was not possible to apply the latest to two animals of the group.

3.2. *Post-mortem* Tests

The results of the *post-mortem* tests for every analyzed animal, calculated sensitivity and its confidence interval are presented next, in Table 15.

Table 15- Results for the *post-mortem* tests applied to the 14 diseased bears and calculated sensitivity (Courtesy of Dr. A. Sha. Arun, Bannerghatta Bear Rescue Centre, Wildlife SOS).

Test	**Gross Pathology**	**Culture**	**PCR**
Sample material	-	Lung Nodule/Lymph Nodes	
Bear 1[47]	NA	NA	NA
Bear 2	P	P	P
Bear 3	P	P	P
Bear 4	P	P	P
Bear 5	P	N	P
Bear 6	P	N	P
Bear 7	P	P	P
Bear 8	P	P	P
Bear 9	P	P	P
Bear 10	P	P	P
Bear 11	P	N	P
Bear 12	P	P	P
Bear 13	P	N	P
Bear 14	P	P	P
Bear 15	P	N	P
Total	**14/14**	**9/14**	**14/14**
Sensitivity (%)	**100**	**64.3**	**100**
Confidence Interval (%)	**68-100**	**35-87**	**68-100**

NA- Not Available; ND- Not Done; P- Positive; N- Negative

During *post-mortem* examinations, lung nodules suggestive of tuberculosis were visually detected in all 14 diseased bears. From these nodules, and draining lymph nodes, *Mycobacterium tuberculosis* was isolated and identified by culture in Lowenstein-Jensen medium in 9 out of these 14 corpses. PCR obtained 100% of positive results, even in the 5 cases that had previously turned out with a negative culture result.

[47] Live bear. No *post-mortem* tests performed.

3.3. *Ante-mortem* Tests Combination

3.3.1. Combination of two different tests

3.3.1.1. Inclusion Criteria

Direct Test: AFB Zeihl-Neelsen Staining (as *ante-mortem* culture and PCR showed a sensitivity of 0% in this study, they will not be included in the combination phase).
Indirect CMI Tests: QuantiFERON-TB Gold® (as TST displayed a sensitivity of 100% it will not be included in the combination phase as any test combined with it would also display 100% sensitivity since a parallel testing approach was applied).
Indirect Serological Tests: ElephantTB STAT-PAK®, DPP VetTB®, Wild TB alert kit®.

3.3.1.2. Results

All the tests selected before were combined in sets of two. For an animal to be considered TB positive, it had to display at least one positive result out of two tests (parallel testing).

Table 16- Sensitivity comparison for each potential combination of two TB screening methods in sloth bears (*Melursus ursinus).*

Test Combination	AFB-ZN Staining	QuantiFERON-TB Gold®	ElephantTB STAT-PAK®	Wild TB alert kit®
DPP VetTB®[48]	8/13	11/13	9/13	12/13
	61.5%	84.6%	69.2%	92.3%
Wild TB alert kit®	13/15	14/15	14/15	
	86.7%	93.3%	93.3%	
Elephant STAT-PAK®	12/15	12/15		
	80%	80%		
QuantiFERON-TB Gold®	10/15			
	66.7 %			

▭- Animals considered positive among the group; ☐ - Sensitivity of the combination.

After making several test combinations, the three combinations with the highest sensitivities, and above 90%, (93.3% and 92.3%), were the ones that combined the Wild TB alert kit® with the ElephantTB STAT-PAK, the QuantiFERON-TB Gold® and the DPP VetTB®. Immediately following, we have the combination of the Wild TB alert kit® with the ZN staining (86.7%).

[48] As previously indicated, the DPP VetTB® test was only performed in 13 animals, thus, only those bears were considered upon the combination phase.

3.3.2. Combination of Three Different Tests

3.3.2.1. Inclusion Criteria

Direct Test: AFB- Zeihl-Neelsen Staining.
Indirect CMI Tests: QuantiFERON-TB Gold®.
Indirect Serological Tests: Wild TB alert kit® (as this was the serological test with the highest sensitivity, it will be the one selected among all serological assays to be combined with the direct and CMI assays).

3.3.2.2. Results

The three tests previously selected were combined. For an animal to be considered TB positive, it had to display either just one positive result or at least two positive results out of the three tests.

Table 17- Calculated sensitivity of the combination of the three categories of tests.

Test	AFB- ZN Staining	QuantiFERON-TB Gold®	Wild TB alert kit®
Bear 1	N	P	P
Bear 2	N	P	N
Bear 3	N	P	P
Bear 4	P	N	P
Bear 5	P	P	P
Bear 6	P	N	P
Bear 7	N	N	P
Bear 8	P	P	N
Bear 9	N	N	P
Bear 10	N	P	P
Bear 11	N	P	P
Bear 12	N	N	P
Bear 13	N	N	N
Bear 14	N	N	P
Bear 15	P	P	P
Total	**5/15**	**8/15**	**12/15**

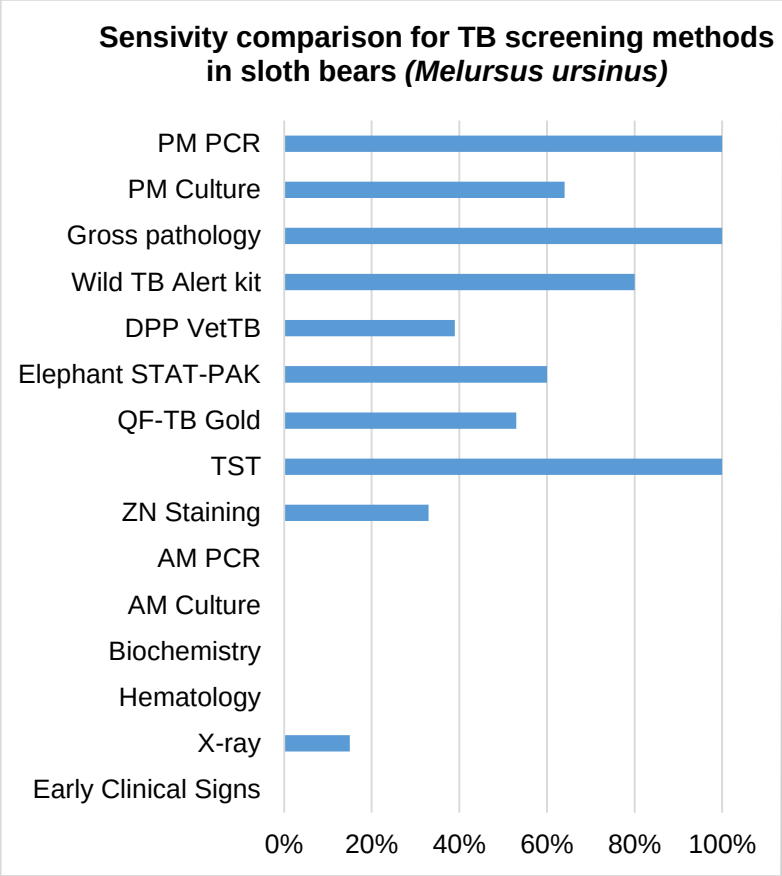
ND- Not Done; P- Positive; N- Negative

Considering an animal to be positive when at least two out of the three are positive, we obtain a sensitivity of 60% (9 in 15). If only one of the three testing modalities had to be positive, then the sensitivity increased further to 93.3% (14 in 15).

3.4. Graphical Analysis

The sensitivity results for all the screening methods analyzed in this study are presented in Graphic 1, providing a facilitated analysis of the same.

Graphic 1- Sensitivity comparison for all the tests applied to the specimen.



PM- post-mortem; AM- ante-mortem

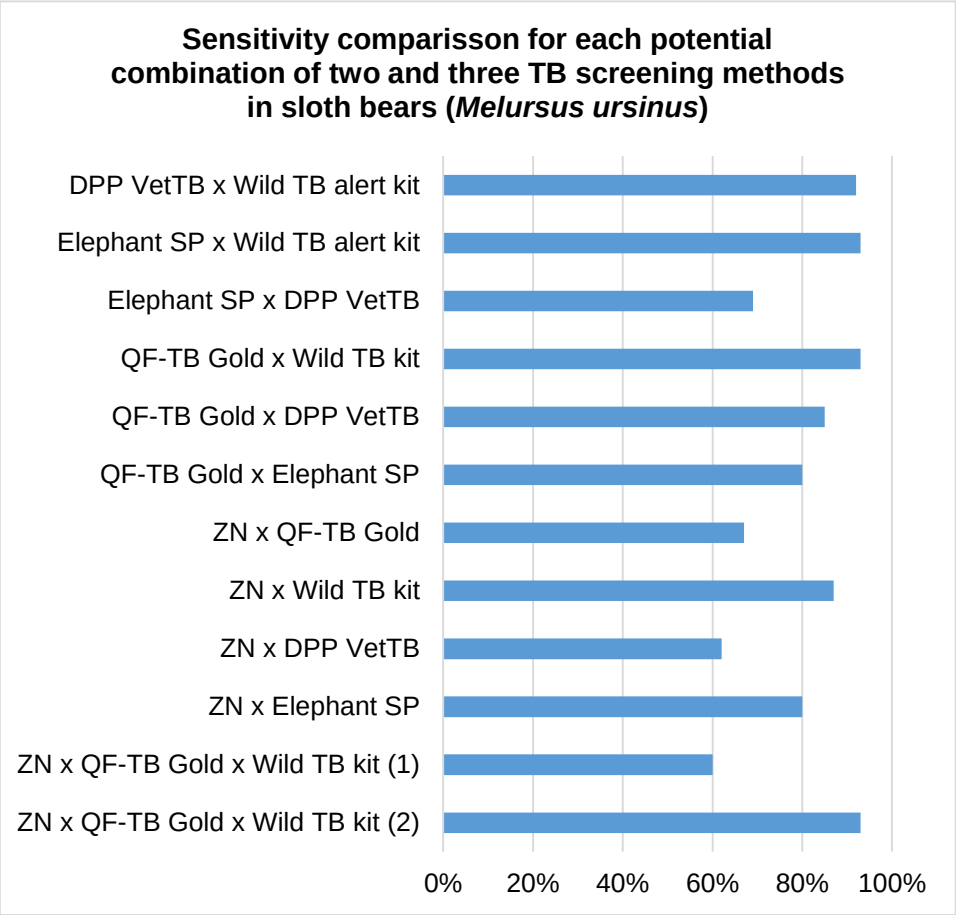
Looking at the graphic display of sensitivity results for the analyzed tests, we can easily infer that *post-mortem* methods (first three from the top) were, in general, the ones with the best results (all sensitivities above 60%), coupled with the tuberculin skin test (100%).

The unspecific tests category (first four from the bottom) showed mostly sensitivities of 0%, with the exception of the thoracic radiography, and staying, overall, below de 20% sensitivity values. Equally disappointing were the sensitivities for *ante-mortem* culture and PCR (0%).

Indirect tests (CMI and serological) displayed a wide range of sensitivity values, from 38.5% (DPP VetTB®) to 100% (TST).

The sensitivity results for all the combinations of tests analyzed in this study are presented in Graphic 2, providing a facilitated analysis of the same.

Graphic 2- Sensitivity comparison for each potential combination of two and three tests.



Graphically translating the sensitivity results for the double combination of diagnostic tests, we can see that, out of 10 different double combinations present, 7 showed a sensitivity value equal to, or above, 80%. Just by analyzing this graphic, we can state that all the combinations of two tests, improved, as expected, the sensitivity the tests displayed singularly.

As for the combination of three tests (last two in the graphic list), we obtained a higher sensitivity value when we considered an animal to be positive when at least one of the three testing modalities had a positive result than if we considered an animal to be positive when at least two of the three had a positive result.

4. Discussion

4.1. General Considerations

As in many other species, to diagnose tuberculosis infection in sloth bears is very challenging, especially prior to their death. In this study, the *post-mortem* tests were, by far, the ones with the highest sensitivity, comparing to the *ante-mortem* tests, in general, although some of the latest seem to show a better correlation with *post-mortem* than others.

In this discussion, the tests will be divided only by the three general categories (unspecific, direct and indirect), in order to simplify the analysis of the same.

In order to determine the sensitivity and specificity for the screening techniques described, some assumptions are required, namely, and as mentioned earlier, that an animal is beforehand considered a positive case when several tests previously taken, revealed a positive result. This way, taking the assumption above, there were no negative animals within this study group, leading to an inability to determine hypothetical specificities for the tests (i.e., there were no true negative or false positive cases).

The addition of control animals to this group would improve the reliability of this diagnostic strategies study but, unfortunately, the lack of this information is justified by the obvious economic restrictions regarding a non-governmental rescue centre in India. If an animal displays more than a negative test multiple times, and it is firmly believed it to be a TB negative individual, in the future, only routine screenings (usually using rapid tests) will be done on the same to check on its health status. It is not viable to apply all the available tests in these apparently negative animals as a routine, but it would definitely be an interesting topic to invest in the future, as it is the only possible way to achieve a full knowledge of the full potential of all the tests in this species.

Another consideration is the limitation of the tests in that they are not specifically designed for this species, and the fact that the time of infection and duration of the same were unknown. All the animals differed in age, year they entered the centre, and length of time they lived within the Kalandar community. Therefore, to make a correct comparison of the tests in question, it is required knowledge of the time infection occurred, which, naturally, was not possible to achieve at all. This is a major limitation in predicting the sensitivities as it is possible that infection occurred earlier and that the tests failed to pick up the infection or presence of *M. tuberculosis*. This is one of the biggest challenges facing infectious disease testing in the clinical setting. Also, in this specific analysis, it is unknown the moment the tests in question were applied to the animals, meaning they could have been used at any time in the bears' lives and not necessarily at the same moment, which represents a significant limitation when drawing conclusions from the tests results.

None the less, based on this data, and accepting the assumptions above, the following can be said of the different testing modalities used in this study:

4.2. Unspecific Tests (*Ante-mortem* and *Post-mortem*)

As it is described multiple times in literature (de Lisle et al., 2002; Backues, 2008), clinical signs for tuberculosis infection are typically nonspecific or absent (at all or until the disease is well advanced) in the majority of infected animals, which we were able to verify, since none of the bears in this study (0%) showed any clinical signs at early stages.

Out of the 13 bears that were submitted to radiography, only 2 (15.4%), showed radiographic signs that matched the image of tuberculous lesions in the lung parenchyma, even though, as mentioned, they did not show any early respiratory clinical signs. Radiographic techniques only provide information regarding the location and aspect of the lesions (Lécu & Ball, 2011), not being very specific (lesions with other etiology may resemble TB lesions) nor sensitive (non-calcified tuberculous lesions or too subtle ones may be missed). Calcified lesions are the ones that are better detected by radiography, although they do not always occur in bears (A.S. Arun, personal communication, April 20th, 2016). In fact, a lot of other animal species generally do not show any calcified lesions (EAZWV, Tuberculosis Working Group, 2010). This way, we can consider radiography an unreliable method, with humans probably being the only species on which is logical to apply this technique.

Also, laboratory analysis of the bears' blood was inconclusive and unspecific to diagnose *Mycobacterium tuberculosis* infection, with the only inconsistent and unremarkable presence of leukocytosis with neutrophilia. No other abnormality worth of mention was detected. This parameter would only be taking into account and considered diagnosing if more remarkable signs were noticed as anemia, lymphopenia, market leukocytosis with neutrophilia and increment of liver enzymes.

At *post-mortem* examinations (necropsy), lesions highly suggestive of tuberculosis were detected, especially within the lungs, draining lymph nodes, and surrounding thoracic area, in all 14 deceased animals, with a 100% sensitivity (naturally taking into account that this is merely a macroscopic visual analysis and the presence of the actual organism must be confirmed by more accurate methods).

Taking the previous statements, *ante-mortem* unspecific diagnostic methods seem to be highly unreliable and should not be used as a standard. They may be used coupled with other methods, especially when we refer to diagnostic strategies as detection of clinical signs and blood analysis, as these exams are always part of the routine animal general health examination, and they do not require an extra effort nor use of resources. Chest X-rays, at least in bears, are highly impractical and do not pay off the use of all the resources that are implicated with the animals' sedation, transportation, staff mobilization and use of the equipment involved[49].

[49] At the time this study was conducted, all X-rays took place at the BBP Zoological Garden, as mentioned in page 62.

Histopathology of affected organs could also have been performed, as sloth bears' lungs are often completely filled with lesions with high potential for an accurate microscopic diagnostic confirmation.

4.3. Direct Tests (*Ante-mortem* and *Post-mortem*)

The direct tests are considered the most reliable way to diagnose tuberculosis infection, as the organism is directly detected or observed, decreasing the probability of occurrence of false positive results (Miller, 2008; Lécu & Ball, 2011; Maas et al., 2013).

Many human diagnostic laboratories resort to culture and PCR techniques combined (Lécu & Ball, 2011), as they represent a very strong and secure diagnosis tool, not only for the detection of mycobacterial infections but also to classify the species of mycobacteria involved (Lécu & Ball, 2011; Maas et al., 2013). PCR techniques allow faster results comparing to the 6-12 weeks that take for culture to reveal bacterial colonies but the latest is still considered the ultimate "gold standard" and a simple way to differentiate between mycobacterial species (Miller, 2008).

Based on experience, it was expected beforehand that *post-mortem* samples would achieve better results compared to the *ante-mortem* ones (Mikota, 2008; Maas et al., 2013) and so it was: culture and PCR of *ante-mortem* samples revealed a sensitivity of 0%, detecting none of the positive animals. On the contrary, at *post-mortem*, culture detected 64.3% of positive cases and PCR detected all 15 of them.

This major discrepancy might be justified by the sample material used in each of the two moments. Sputum collected *ante-mortem*, as in other species, is poorly sensitive in diagnosing mycobacterial infection, as animals with active tuberculosis are thought to shed bacteria for limited periods of time (intermittent shedding) (Miller, 2008; Lécu & Ball, 2014). Other limitations, regarding the sample collection, and that may have affected the amount of bacilli detected, are the occurrence of highly calcified lesions (though these lesions were only radiographically detected in 2 bears) (Capuano et al., 2003), site of infection and pulmonary cavitation cases. Bacilli may also not be present in a particular sputum sample, or even be lost in the decontamination treatment required to process sputum for mycobacterial culture (Frieden et al., 2003; Guillerm et al., 2006). Other possible explanations for the lack of positive findings, are obviously related to human errors, either at sample collection, sample accommodation, sample processing and testing procedures.

Also, in the possibility that these methods were performed after treatment, the presence of the organism in the specimens might have been reduced or even absent.

Post-mortem results were much more pleasant, most likely because the sample materials for analysis were collected directly from tuberculous lesions (nodules in lungs) and lymph nodes. In a study regarding *Mycobacterium pinnipedi* infection in sea lions, post-treatment euthanized animals revealed the presence of mycobacteria in calcified lymph nodes but only with the use of PCR. Culture results remained negative (Jurczynski, 2012). Similarly, Iida et al. (2014) reported

that granulomatous lesions with calcification in humans, contained considerable amounts of *M. tuberculosis* genomes and cell wall components and, despite exhibiting little inflammation, were all positive for *M. tuberculosis* by polymerase chain reaction and uniformly negative by culture. This suggests that PCR might display a higher sensitivity comparing to culture when dealing with calcified lesions, which could justify the difference between the two methods' results in the sloth bears' case. Also, and again, the possibility of human errors should be taking into account for the negative culture results.

As culture is unlikely to yield a false-positive (Mikota, 2008) we can infer that while a positive culture provides evidence of disease, a negative culture result may not rule out infection in exposed or suspect animals (Lécu & Ball, 2011; Maslow & Mikota, 2015). On the other hand, diagnostic techniques based on PCR might yield false-positive reactions due to contamination with DNA fragments from previous PCRs and false-negative reactions caused by inhibitors that interfere with the PCR (Kox et al., 1994).

The Ziehl-Neelsen Staining technique was able to detect 5 positive animals out of 15 (33.3%). There may be some difficulty in detecting acid-fast bacilli (AFB) using this method, as at least 1.000 organisms/mL are necessary, yielding a low sensitivity of microscopy (Lécu & Ball, 2011) and, again, sputum was the material used. This technique could have been used at *post-mortem* as well, as the amount of bacilli detected would likely be higher, increasing the chances of a positive diagnosis. However, it must be taken into account that all mycobacterial species are acid-fast, which means that this method is unable to distinguish between tuberculous and nontuberculous mycobacteria (including environmental mycobacteria) (Somoskovi et al., 2008).

4.4. Indirect Tests

The CMI tests can be divided into two groups, based on their application: *in vivo* (TST) and *in vitro* (IFN-γ).

The Tuberculin Skin Test (*in vivo*) showed positive results in all 9 bears that were submitted to it, with reactions that were considered undoubtedly positive (6-8mm) after evaluation, with a sensitivity of 100%. However, this method is not currently used as a standard at BBRC, being considered unreliable and not differentiating between a latent infection and an active disease. Also, because it requires two different moments of sedation, being highly impractical and expensive, it is mostly avoided. Nevertheless, there is no doubt that this test proved the ability to detect all 9 positive animals submitted to it. As an alternative, and as the test is performed on the ear lobe and sloth bears proved to be capable of being trained, it might be possible to train the animals to be kept still, positioning their heads in a way that allows the ear to stick out of the enclosure, for reaction examination and measurement. This way, the injection would take place in a routine sedation moment (and the human operator would be able to ensure the proper execution of the procedure, without the risk of a possible movement from the animal), and the

reading could happen 72 hours later, in a more stress-free animal environment, and without the need of anesthesia and all the risks and costs regarding it.

Moreover, the potential infection by other mycobacterial species should not be over looked and it might be profitable, if TST would ever be used as a standard method again, to implement an intradermal test with PPD tuberculins prepared from *M. tuberculosis* and *M. avium*, for single and comparative testing.

The QuantiFERON-TB Gold®, was able to obtain a 53.3% sensitivity, as 8 bears among the 15 showed positive results. Like the skin test, the IFN-γ release assay has time limits caused by decreasing CMI responsiveness during the disease progression (Maas et al., 2003) which can explain the sensitivity value obtained, that was not promising enough to think of it as an ideal test. Another limitation of this method is the fact that requires blood samples to be processed within 8-30 hours after collection, which largely restricts its use, and potential errors in collecting or transporting the blood specimens or in running and interpreting the assay can decrease the accuracy of IGRAS (Interferon-gamma release assays) (Mazurek et al., 2011).

In wildlife medicine, IGRAS have the advantage of requiring only one handling of the animals (Mikota et al., 2000; de Lisle et al., 2002), and, being *in vitro* tests, prevent some of the potential variability of *in vivo* tests, being also very effective for detecting latent infections (Miller, 2008; Lécu & Ball, 2014).

The serological results helped to identify most of the infected sloth bears, though with some notorious differences. ElephantTB STAT-PAK® assay, Wild TB alert kit® and the DPP VetTB® assay were, overall, useful point-of-care tools to detect seroconversions and identify the presence of antibodies. Furthermore, the Wild TB Alert Kit® showed the highest sensitivity (80%) compared to ElephantTB STAT-PAK® assay (60%) and DPP Vet assay® (38.5%), suggesting its greatest agreement with the necropsy findings. This tests have the great advantages of being easy to use, cheap and very fast in revealing results (Lyashchenko et al., 2000; Achkar et al., 2011).

4.5. *Ante-mortem* Tests Combination

The available diagnostic techniques are not as reliable as stand-alone tools as they are combined. When pairing up together the most fitting screening methods, we were able to increase the sensitivity the methods displayed singularly, resorting to a parallel testing approach. The two combinations that obtained the highest sensitivity (93.3%) were the ones that combined the Wild TB alert kit® with both the Elephant STAT-PAK and the QuantiFERON-TB Gold®, followed by the combination of the Wild TB alert kit® with the DPP VetTB® (92.3%), and by the pairing of the Wild TB alert kit® with the ZN Staining (86.7%). Every set of two tests surpassed the sensitivity that each test revealed alone. Many literature cases state that combinations of tests always improve the accuracy of TB diagnosis, as long as parallel tests that are independent from each other are chosen (Guillerm et al., 2006; Miller, 2008; Lécu & Ball, 2014).

It was also experimented to combine three different tests of the three major specific categories (direct, CMI and serological) as this is considered the wisest approach to achieve a reliable diagnose in species on which there is no specific nor validated tests (EAZWV Tuberculosis Working Group, 2010; Lécu & Ball, 2011). If we consider an animal to be positive when at least two out of the three (AFB- ZN Staining, QuantiFERON-TB Gold® and Wild TB alert kit®) are positive, we obtain a sensitivity of 60% (9 in 15), which is higher than the sensitivity of AFB- ZN Staining and QuantiFERON-TB Gold® alone but considerably lower than the Wild TB alert kit® sensitivity by itself (80%). If only one out of the three testing modalities had to be positive, then the sensitivity would increase further to 93.3%.

After the results' analysis, the highest sensitivity achieved, either by combining two or three tests was of 93.3%, by parallel testing. This way, and based on this study, we can assess that we have no advantages in combining three modalities of tests instead of two, in terms of sensitivity increment.

The interpretation of "one out of three" as a positive is useful in a screen program for which the risk of a positive animal to a collection is considered high. Considering the former "two out of three" tests as a positive allows a more ethical approach to managing the animals in a collection. However, it comes with the consideration that positive animals may be missed or considered negative.

4.6. *Ante-mortem* Testing Strategies

As mentioned earlier, the combination of all three specific categories (direct, CMI and serology) would be the most ideal approach for a secure and accurate diagnosis, even though they obtained, combined, the same sensitivity value as combinations of two tests, in this study. Direct tests would be directed to infer the animal's organism shedding status as the use of indirect tests, indicated for both phases of the host immunologic response, would be the most suitable way to avoid failing the detection of positive animals, as they provide a wider diagnostic range.

In the sloth bears' case, and considering this ideal scenario, the direct test of choice in this study would be the ZN staining as it was the only one among this particular group of tests with positive results, even though culture is theoretically the method that carries more advantages. The CMI based test of choice would most likely be the TST[50], as IGRAS require blood samples to be processed within hours after collection, which is quite difficult to accomplish by wildlife centres, usually located far from the laboratory that analyzes the samples, and, naturally, because TST was able to detect 100% of positive animals in this study. Finally, the serological rapid test of choice, given its greater results comparing to the remaining ones, would be the Wild TB alert kit®.

[50] TST was not the elected test in the previously discussed "combination of three tests" only because the main goal then was to infer how the sensitivity of the tests would increase, once they were combined. As the sensitivity of this particular test was 100% to begin with, its use would only result in an 100% combination sensitivity, missing the initial point of the combination.

Nonetheless, the so called “ideal scenario” is not always possible to apply, especially in resource limited settings. For this kind of realities, there must be alternatives and strategies adapted to them. With this in mind, and after evaluating all the different results and combinations, it was possible to assess five potential different approaches taking into account economic restrictions, animals’ welfare and animals’ immune response to TB infection:

1. Using a CMI based test (TST or QuantiFERON-TB Gold®) and serology (Wild TB alert kit®): ideally, this would be the most secure and reliable approach, as each of these categories of tests are indicated for each phase of the host immune response (within the progressive course of the disease, the cellular immune response decreases and the humoral immune response increases), creating a wider diagnostic range. Moreover, CMI based tests, which exploit T cell responses, are highly effective for detecting latent infections (Lécu & Ball, 2014). TST is considerable less expensive that the QuantiFERON-TB Gold®, does not come with the urgency of processing samples within a few hours after collection and achieved a higher sensitivity value in this study (100%). On the other hand, the interferon-gamma release assay has the advantage of requiring only one handling of the animals, it avoids some of the potential variability of *in vivo* tests, as it is performed in a laboratory, and its combination with Wild TB alert kit® was one of the two that achieved the highest sensitivity by parallel testing (93.3%).

2. Using serology (Wild TB alert kit®) and microscopy (ZN staining): As previously stated, any repeated positive outcome in the immunological tests should lead to an investigation of the direct exam category, in order to determine the shedding status of the animal (EAZWV Tuberculosis Working Group, 2010; Lécu & Ball, 2011). Although this was not a combination that achieved one of the highest sensitivity values (86.7%), the advantages in using two methods from the direct and indirect categories, should be taking into account (one is based on the organism detection and the other on the host’s immune response to it, improving the chances of an accurate diagnosis) (Lécu & Ball, 2011), and for that reason, this is the combination described in second place. Also, microscopy using the Ziehl-Neelsen method is the fastest, easiest and least expensive method of TB direct diagnosis and should not be under looked (Mikota. 2008; Rishikesava et al., 2008; Somoskovi, Gutierrez & Salfinger, 2008). In fact, although culture is considered the “gold standard” for the bacteriologic diagnosis of TB, many times, in resource-limited countries, diagnosis is often based solely on sputum smear microscopy results (van der Kuyp & Mahan, 2012). However, it must be taken into account that this method is unable to distinguish between tuberculous and nontuberculous mycobacteria (including environmental mycobacteria), thus being essential that is always used combined with other methods (Somoskovi et al., 2008);

3. Using two serological tests (with one of them being the Wild TB alert kit®): most TB positive animals that reach BBRC are assumed to be infected for some time, since they cohabit

with humans from a young age. This way, and considering that the serological rapid tests are relatively inexpensive and easy to use, the choice of this combination (93.3%/92.3% sensitivity) is a rather clever and profitable approach, as serological tests are not useful in early stages of infection but become very effective with the progression of the same (Lécu & Ball, 2011). Nevertheless, and despite years of effort and research, serological tests are not considered the ideal ones to detect latent infections (Achkar et al., 2011), as, in latency, the organism is usually "hidden" from the immune system, inside macrophages (Olsen et al., 2010). This way, by only using this kind of methods as a standard, there is a high risk of under detection of positive animals. This said, it is always advisable to correlate with a CMI based method and/or a direct assay;

4. Using only the TST: based on this study, this was the test that achieved the highest sensitivity (100%), hence, in theory, the most reliable one to detect positive animals, and with a high possibility of being taken as a standard method, given its ability to detect latent infections (Achkar et al., 2011). Nevertheless, presently, at BBRC, this method implicates the existence of a second sedation and everything related to it (staff mobilization, use of sedation drugs and putting the animal through a stressful situation for the second time, with only 3 days apart), to evaluate the reaction to the injection, being the method that shows the lowest animal welfare consideration and also not being the most economically viable. Also, we must be reminded that this method can be affected by immunosuppressive status, anergic status and exposure to environmental mycobacteria, being, as well, unable to differentiate between latent and active infections and having time limits caused by decreasing CMI responsiveness during the disease progression (Mikota et al., 2000; de Lisle et al., 2002; Maas et al., 2003; Mikota, 2008; Lécu & Ball, 2011; Maas et al., 2013). Therefore, even though the sensitivity of this test is undeniably high, all the factors that can interfere with it, given the fact that is an *in vivo* assay with high variability, would probably result in a not such great value for specificity, although more investigations regarding this matter should be made in order to make this statement;

5. Using only the Wild TB alert kit®: as this was the test that showed the second highest sensitivity by itself (80%), only implicates one animal sedation and, being a rapid test, is fast, easy to use and economically accessible, it would probably be the most indicate choice for extremely resource-limited settings and could ultimately serve as a point-of-care test (Achkar et al., 2011), though it should not be forgotten that an indirect test alone is probably meaningless if it is not combined with direct screenings and, if possible, with another indirect assay (Miller, 2008; Lécu & Ball, 2014). Also, as mentioned in approach number 3, serological tests are not considered the ideal ones to detect latent infections (Achkar et al., 2011).

As with other species, tuberculosis testing and its correspondent interpretation always require assessment of the history, knowledge of exposure to mycobacteria and management decisions for a particular cohort, in combination with disease risk analysis for each individual situation.

5. Conclusions

Tuberculosis infection in captive sloth bears is an ongoing and complex problematic occurring in an under looked wild species in an under looked country. Due to the nature of this disease, the diagnosis is rather complicated, apart from the fact that most tests developed for domestic species have not been validated for exotic animals and, therefore, may have sub-optimal sensitivity and/or specificity. This said, it is of major importance to designate, among the available tests, which ones are the most accurate for sloth bears, giving veterinarians a standardized methodology that will allow them to take preventive measures and establish early contention and treatment protocols, as we are dealing with (1) a zoonosis that affects one third of the world's human population and needs to be urgently contained; (2) a protected animal species in which euthanasia is not an option. Moreover, it is highly important for the veterinary community that the diagnostic methods applied on an animal cohort, no matter the species, are consistent and methodical, especially in a disease as unpredictable as tuberculosis in both organism excretion/detection and progression of the host's immune response. As for now, the diagnostic methods applied are hardly homogeneous among and within species.

Depending on interpretation, and/or its combination, the tests presented in this dissertation can be used to provide a strong evidence of *M. tuberculosis* infection. This small study highlights the potential usefulness and efficacy of indirect and direct *ante-mortem* diagnostic methods, proving that the best strategy to achieve high sensitivity in tuberculosis detection in sloth bears and other animals is the use of multiple diagnostic methods (preferably from distinct categories) rather than a single test. Nevertheless, it is important to keep in mind that an evaluation and validation of this testing regime will require the addition of more animals into the cohort.

The indirect assays evaluated in this study (rapid serological tests as ElephantTB STAT-PAK® and Wild TB alert kit® and CMI based tests as the TST and QuantiFERON-TB Gold®), showed particularly encouraging results, especially when combined, and should be considered as very important and reliable tools to detect TB positive sloth bears *ante-mortem*. However, serological techniques may have an even higher potential value as they are cheap, simple, rapid, relatively noninvasive, and can be accurate as long as appropriate antigens and immunoassay formats are used.

Complete *post-mortem* examinations must always be performed along with cultures, PCR, and histopathological examination, as many *ante-mortem* screenings fail to reveal all of the positive animals and *post-mortem* techniques showed a sensitivity that was around 100% in this study.

Moreover, it is important to keep an open mind, and be reminded that sloth bears can virtually be affected by several mycobacterial species. The search for other sources of infection should not be under looked, as "dancing bears" also contact with cattle in their human domestic environment, and free-ranging rescued bears potentially may have had contact with several maintenance hosts in the wild.

The main goals established for this study were accomplished. However, as it was not possible to calculate the specificity of the diagnostic tests in question, we are still lacking a global evaluation of the same and their full potential. The addition of control animals (confirmed negative) to the cohort would improve the accuracy of the tests individual evaluation.

Other limitations regarding this study that should be taking into account when drawing conclusions is the fact that the time of infection and duration of the same were unknown, as well as the moment the tests in question were applied to the animals.

Major and deeper investigations in this area are urgently needed. Alternative treatments, specific for animals, should be pursued and tested in order to reduce the use of treatment with human drugs and their consequent side effects. Apart from that, pharmacokinetic studies are fundamental to know the optimal concentration of tuberculosis treatment options, and these are still lacking for sloth bears. Also and mostly, development and testing of new and more specific methods of diagnosis is the need of the moment, as it would increase the possibility of an accurate diagnosis that might permit an early, and thus most likely effective, treatment and application of preventive measures that will ensure the safety of the remaining animals and human staff.

The long incubation periods and latency of any mycobacterial disease also require that surveillance plans are established on a long-term basis in order to be effective.

Results and conclusions from this study will hopefully allow a better understanding of the sloth bear tuberculosis control reality present in Indian rescue centres and contribute with data that can be used by other rescue centres and zoological collections in their sloth bear groups. Nevertheless, given the lack of information, studies and methodology regarding *M. tuberculosis* infection in wildlife animals in general, this work will hopefully also be of some use and insight for other species, as many more contributed for this one.

References

Abebe, F., Holm-Hansen, C., Wiker, H.G., Bjune, G. (2007). Progress of Serodiagnosis of Mycobacterium tuberculosis infection. *Scandinavian Journal of Immunology*, 66, 176-191.

Achkar, J.M., Lawn, S.D., Moosa, M.S., Wright, C.A. &. Kasprowicz, V.O. (2011). Adjunctive Tests for Diagnosis of Tuberculosis: Serology, ELISPOT for Site-Specific Lymphocytes, Urinary Lipoarabinomannan, String Test, and Fine Needle Aspiration. *The Journal of Infectious Diseases* 2011(204), 1130–1141.

Alexander, K.A., Pleydell, E., Williams, M.C., Lane, E.P., Nyange, J.F.C., & Michel, A.L. (2002). *Mycobacterium tuberculosis*: An Emerging Disease of Free-Ranging Wildlife. *Emerging Infectious Diseases*, 8(6), 598–601.

Allen, A.R., Minozzi, G., Glass, E.J., Skuce, R.A., McDowell, S.W. J., Woolliams, J.A. & Bishop, S.C. (2010). Bovine tuberculosis: the genetic basis of host susceptibility. *Proceedings of the Royal Society B: Biological Sciences*, 277, 2737–2745.

Angkawanish, T., Wajjwalku, W., Sirimalaisuwan, A., Kaewsakhorn, T., Boonsri, K., Rutten, V., Mahasawangkul, S. (2010). *Mycobacterium tuberculosis* infection of domesticated Asian elephants, Thailand. *Emerging Infectious Diseases*, 16(12), 1949–1951.

Angkawanish, T., Morar, D., van Kooten, P., Bontekoning, I., Schreuder, J., Maas, M., Wajjwalku, W., Sirimalaisuwan, A., Michel, A., Tijhaar, E. & Rutten, V. (2013). The Elephant Interferon Gamma Assay: A Contribution to Diagnosis of Tuberculosis in Elephants. *Transboundary and Emerging Diseases*, 60: 53–59.

Arun A.S., Kajal J.K., Selvaraj, I. & Selvaraj, V. (2008). Hematology of sloth bears (*Melursus ursinus ursinus*) from two locations in India. *The Journal of Wildlife Diseases*, 44(2), 509-518.

Arun, A.S., Abraham, D., Selvaraj, I., Khadpekar, Y., Anusree, S. B., Alex, P.C. & Mundayoor, S. (2014). Evaluation of Diagnostic Tests for *ante-mortem* tuberculosis screening in captive sloth bears (*Melurus ursinus*) [abstract] [electronic version]. In C.K. Baer (Ed) *Proceedings of the 46th Annual Conference AAZV, Orlando, Florida, USA*, *18-24 October,* p.160. Accessed on April 25th, 2016, available at https://www.researchgate.net/publication/269047930_EVALUATION_OF_DIAGNOSTIC_TESTS_FOR_ANTE-MORTEM_TUBERCULOSIS_SCREENING_IN_CAPTIVE_SLOTH_BEARS_Melurus_ursinus.

Ayele, W.Y., Neill, S.D., Zinsstag, J., Weiss, M.G., & Pavlik, I. (2004). Bovine tuberculosis: an old disease but a new threat to Africa. *The International Journal of Tuberculosis and Lung Disease*, *8*(8), 924–937.

Backues, K.A. (2008). Chapter 36: Mycobacerial Diseases in Carnivores. In M.E. Fowler, & E.R. Miller (Eds), *Zoo and Wildlife Medicine, Current therapy, Volume 6*. (pp 294-298). London, UK: Elsevier Health Sciences.

Beggins, R.G. (1999). Chapter 16: TB in Free-ranging animals. In M.E. Fowler, & E.R. Miller (Eds), *Zoo and Wildlife Medicine, Current therapy, Volume 4*. (pp 101-114). Philadelphia: Saunders and Company.

Boomershine, C.S. & Zwilling, B.S. (2000). Stress and the Pathogenesis of Tuberculosis. *Clinical Microbiology Newsletter,* 22 (23), 177-182.

Bourne, D.C., Cracknell J.M. & Bacon H.J. (2010). Veterinary issues related to bears (Ursidae) in *International Zoo Yearbook*, 44, 16–32.

Briones, V., De Juan, L., Sanchez, C., Vela, A. I., Galka, M., Montero, N., Goyache, J., Aranaz, A. & Domìnguez, L. (2000). Bovine tuberculosis and the endangered Iberian Lynx. *Emerging Infectious Diseases*, *6*(2), 189–191.

Bruning-Fann, C.S., Schmitt, S.M., Fitzgerald, S.D., Fierke, J.S., Friedrich, P.D., Kaneene, J.B., Clark, K.A., Butler, K.L., Payeur, J.B., Whipple, D.L., Cooley, T.M., Miller, J.M. & Muzo, D.P. (2001). Bovine tuberculosis in free-ranging carnivores from Michigan. *Journal of Wildlife Diseases*, *37*(1), 58–64.

Buddle, B.M., Parlane, N.A., Wedlock, D.N. & Heiser A. (2013). Overview of Vaccination Trials for Control of Tuberculosis in Cattle, Wildlife and Humans. *Transboundary and Emerging Diseases,* 60 (1), 136–146.

Chapel, H., Haeney, M., Misbah, S. & Snowden, N. (2006). Chapter 1: Basic Components: Structure and Function. In H. Chapel, M. Haeney, S. Misbah & N. Snowden (Eds), *Essentials of Clinical Immunology,* (5th Ed.). (pp: 1-32). Oxford, UK: Blackwell Publishing.

Ciftci, E., Ilgazli, A., Gulleroglu, B., Ara, I., & Akansel, G. (2005). Ultrasonographic Measurement of the Tuberculin Skin Test. *Infectious Diseases in Clinical Practice*, *13*(1), 20–23.

Collins, (2014). Chapter 50- Ursidade. In M.E. Fowler, & E.R. Miller (Eds), *Zoo and Wildlife Medicine, Current therapy, Volume 8*. (pp: 498-508). London, UK: Elsevier Health Sciences.

Combs, G.F. (2012). Chapter 13: Vitamin B6. In G. F. Combs (Ed) *The Vitamins: Fundamental Aspects in Nutrition and Health* (4th ed.). (pp: 309- 323). USA: Elsevier/Academic Press.

Cooper, A.M. & J.L. Flynn. (1995). The protective immune response to *Mycobacterium tuberculosis*. *Current Opinion in Immunology*, 7, 512–516.

Da Silva, M.V., Tiburcio, M.G.S., Machado, J.R., Silva, D.A.A., Rodrigues, D.B.R., Rodrigues, V., & Oliveira, C.J.F. (2015). Complexity and Controversies over the Cytokine Profiles of T Helper Cell Subpopulations in Tuberculosis. *Journal of Immunology Research*, 22 (2015), Article 639107. Accessed on July 10th, 2016, available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4606092/pdf/JIR2015-639107.pdf.

Dalovisio, J.R., Stetter, M. & Mikota-Wells, S. (1992) Rhinoceros' rhinorrhea: Cause of an outbreak of infection due to airborne *Mycobacterium bovis* in zookeepers. *Clinical Infectious Diseases*, 154, 598-600.

Dalovisio, J.R., Montenegro-James, S., Kemmerly, S.A., Genre, C.F., Chambers, R., Greer, D., Pankey, G.A., Failla, D.M., Haydel, K.G., Hutchinson, L., Lindley, M.F., Nunez, B.M., Praba, A., Eisenach, K.D. & Coope, E.S. (1996). Comparison of the Amplified *Mycobacterium tuberculosis* (MTB) Direct Test, Amplicor MTB PCR, and IS6110-PCR for Detection of MTB in Respiratory Specimens. *Clinical Infectious Diseases,* 1996(23), 1099-1106.

Davis, M. (2001). *Mycobacterium tuberculosis* risk for elephant handlers and veterinarians. *Applied Occupational and Environmental Hygiene*, 16, 350-353.

D'Cruze, N., Sarma, U.K., Mookerjee, A., Singh, B., Louis, J., Mahapatra, R.P., Jaiswal, V.P., Roy, T.K., Kumari, I. & Menon, V. (2011). Dancing bears in India: A sloth bear status report. *Ursus*, 22(2), 99-105.

De Lisle, G.W., Bengis, R.G., Schmitt, S.M., & O'Brien, D.J. (2002). Tuberculosis in free-ranging wildlife: detection, diagnosis and management. *Revue scientifique et technique (International Office of Epizootics)*, 21(2), 317-334.

Deshpande, P. S., Kashyap, R. S., Ramteke, S. S., Nagdev, K. J., Purohit, H. J., Taori, G. M., & Daginawala, H. F. (2007). Evaluation of the IS6110PCR assay for the rapid diagnosis of tuberculous meningitis. Cerebrospinal Fluid Research, 4(10), 1-7.

Dhabhar, F.S. (2002). Stress-induced augmentation of immune function—the role of stress hormones, leukocyte trafficking, and cytokines. *Brain Behavior and Immunity,* 16, 785–798.

Dhabhar, F. S. (2014). Effects of stress on immune function: The good, the bad, and the beautiful. *Immunologic Research*, 58(2-3), 193–210.

Dietrich, J., Roy, S., Rosenkrands, I., Lindenstrøm, T., Filskov, J., Rasmussen, E. M. & Cassidy, J. (2015). Differential Influence of Nutrient-Starved *Mycobacterium tuberculosis* on Adaptive Immunity Results in Progressive Tuberculosis Disease and Pathology. *Infection and Immunity*, 83 (12), 4731–4739.

EAZWV Tuberculosis Working Group. (2010). Chapter 10: Tuberculosis in Zoo species: diagnostic update and management issues. In EAZWV Infectious Diseases Working Group, *Transmissible Diseases Handbook* (4th ed.). (pp: 96-116).

Ernst, J.D., & Wolf, A. (2006). Chapter 8: *Mycobacterium tuberculosis*: Mechanisms of phagocytosis and intracellular survival. In J.D. Ernst & O. Stendahl (Eds)*, Phagocytosis of Bacteria and Bacterial Pathogenicity, Volume 12 of Advances in Molecular and Cellular Microbiology.* (pp: 246- 265). Cambridge, UK: Cambridge University Press.

Ernst, J.D. (2012). The immunological life cycle of tuberculosis. *Nature Reviews Immunology,* 12(8), 581-591.

Fefar, D.T., Jivani, B.M., Mathukiya, R.A., Undhad, V.V., Ghodasara, D.J., Josh, B.P., Dave, C.J. & Prajapati, K.S. (2012). A case report of tuberculosis in a captive Sloth Bear (*Melursus ursinus*). *Zoo's Print*, 27(2), 25-26.

Fico, R., Mariacher, A., Ciarrocca, E., Eleni, C., Franco, A., Battisti, A. (2015). First Report of *Mycobacterium bovis* infection in a free-ranging Marsican brown bear. In Societa' Italiana delle Scienze Veterinarie (Eds). *Proceedings of the XII Conference AIPVet, Perugia, Italy, 15-17 June 2015. Accessed November 18th, 2016, available at https://www.researchgate.net/publication/278963981_First_Report_of_Mycobacterium_bovis_infection_in_a_free-ranging_Marsican_brown_bear.*

Fitzgerald, S.D., Kaneene, J.B., Butler, K.L. K.L., Clarke, K.R., Fierke, J.S., Schmitt, S.M., Bruning-Fann, Mitchell, R.R., Berry, D.E. & Payeur, J.B. (2000). Comparison of *Post-mortem* Techniques for the Detection of *Mycobacterium bovis* in White-tailed Deer. *Journal of Veterinary Diagnostic Investigation*, 12, 322-327.

Flynn, J. L., Capuano, S. V., Croixa, D., Pawara, S., Myersa, A., Zinovika, A. & Kleinb, E. (2003): Nonhuman primates: a model for tuberculosis research. In Proceedings from the 4th World Congress on Tuberculosis. *Tuberculosis* 83(1–3), 116–118

Fortin, A., Abel, L., Casanova, J.L. & Gros, P. (2007) Host genetics of mycobacterial diseases in mice and men: forward genetic studies of BCG-osis and tuberculosis. *Annual Review of Genomics and Human Genetics*, 8, 163–192.

Fowler, M.E. (1986). Chapter 5: Stress. In M.E. Fowler, & E.R. Miller (Eds), *Zoo and Wildlife Medicine, Current therapy, Volume 2*. (pp 33-37). London, UK: Elsevier Health Sciences.

Gadkowski, L.B & Stout, J.E (2008). Cavitary Pulmonary Disease. *Clinical Microbiology Reviews*, 21 (2), 305–333.

Gamboa, F., Manterola, J. M., Viñado, B., Matas, L., Giménez, M., Lonca, J., Manzano, J.R., Rodrigo, C., Cardona, P.J., Padilla, E., Domínguez, J. & Ausina, V. (1997). Direct detection of *Mycobacterium tuberculosis* complex in nonrespiratory specimens by Gen-Probe Amplified *Mycobacterium Tuberculosis* Direct Test. *Journal of Clinical Microbiology*, 35(1), 307–310.

Garshelis, D.L., Ratnayeke S. & Chauhan, N.P.S. (2008). *The IUCN Red List of Threatened Species: Melursus ursinus*. Accessed on April 21st, 2016, available at http://www.iucnredlist.org/details/13143/0.

Gori, A., Bandera, A., Marchetti, G., Esposti, A.D., Catozzi, L., Nardi, G.P., Gazzola, L., Ferrario, G., van Embden, J.D.A., van Soolingen, D., Moroni, M., & Franzetti, F. (2005). Spoligotyping and *Mycobacterium tuberculosis*. *Emerging Infectious Diseases*, 11(8), 1242-1248.

Grange, J.M., Yates, M.D., de Kantor, I.N. (1996). *Guidelines for Speciation within the Mycobacterium tuberculosis complex*. (2nd ed). Geneva, Switzerland: World Health Organization, Division of Emerging and Other Communicable Diseases Surveillance and Control. Accessed November 16th, 2016, from The World Health Organization website: http://apps.who.int/iris/bitstream/10665/65508/1/WHO_EMC_ZOO_96.4.pdf .

Grange, J.M. (2014). Chapter 3: *Mycobacterium tuberculosis*: The Organism. In P.D.O. Davies, S.B. Gordon, G. Davies (Eds), *Clinical Tuberculosis*. (5th ed). (pp: 39-50). Boca Raton, Florida: CRC Press.

Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J. H., Lyashchenko, K. P., Ball, R., Dumonceaux, G., Schmitt, D., Moller, T., Payeur, J.B., Harris, B., Sofranko, D., W. Ray Waters, W.R. & Lyashchenko, K.P. (2009). Highly Accurate Antibody Assays for Early and Rapid Detection of Tuberculosis in African and Asian Elephants. *Clinical and Vaccine Immunology,* 16(5), 605–612.

Guillerm, M., Usdin, M. & Arkinstal, J. (2006). *Tuberculosis Diagnosis and Drug Sensitivity Testing: An overview of the current diagnostic pipeline*. Accessed on April 16th, 2016, available at http://fieldresearch.msf.org/msf/handle/10144/13532.

Helke, K.L., Mankowskia, J.L. & Manabe, Y.C. (2006). Animal models of cavitation in pulmonary tuberculosis. *Tuberculosis,* 86(5), 337-348.

Holdiness MR. (1984). Clinical pharmacokinetics of the antituberculosis drugs. *Clinical Pharmacokinetics,* 9, 511-44.

Houston, S. & Fanning, A. (1994). Current and potential treatment of tuberculosis. *Drugs*, 48(5): 689-708.

Iallegio, D.M. (1997). Mycobacterial Disease in the Nonhuman Primate. *Seminars in Avian and Exotic Pet Medicine*, 6 (1), 34-39.

Iida, T., Uchida, K., Lokman, N., Furukawa, A., Suzuki, Y., Kumasaka, T., Takemura, T., Kawachi, H., Akashi, T. & Eishi, Y. (2014). Calcified Granulomatous Lung Lesions Contain Abundant *Mycobacterium tuberculosis* Components. *Journal of Mycobacterial Diseases*, 4(1), 1-6.

Isaza, R. (2003). Chapter 66: Tuberculosis in all *taxa*. In M.E. Fowler, & E.R. Miller (Eds), *Zoo and Wildlife Medicine, Current therapy, Volume 5*. (pp: 689-696). London, UK: Elsevier Health Sciences.

IUCN Red List of Threatened Species. (2008). *Bear species conservation status*. Key word searched: "Ursidae". Accessed: April 25th 2016, available at http://www.iucnredlist.org/search.

Joint United Nations Programme on HIV/AIDS (UNAIDS). (2016). *Global AIDS Update 2016*. Acessed November 16th, 2016, available at http://www.unaids.org/en/resources/documents/2016/Global-AIDS-update-2016.

Jurczynski, K., Lyashchenko, K.P., Scharpegge, J., Fluegger, M., Lacave, G., Moser, I., Tortschanoff, S. & Greenwald, R. (2012). Use of Multiple Diagnostic Tests to Detect *Mycobacterium pinnipedii* Infections in a Large Group of South American Sea Lions *(Otaria flavescens*). *Aquatic Mammals*, 38(1), 43-55.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten A., Molhuizen, H., Shaw, R., Goyal, M. & van Embden J. (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *Journal of Clinical Microbiology*, *35*(4), 907–914.

Kaneene, J.B. & Pfeiffer, D. (2006) Epidemiology of *Mycobacterium bovis*. In C. O. Thoen, J. H. Steele, M. J. Gilsdorf. *Mycobacterium Bovis Infection in Animals and Humans*. (2nd ed). pp: 345-345 Oxford, UK: Blackwell Publishing Ltd.

Kelley, K. W. (1980). Stress and immune function: a bibliographic review. *Annales de Recherches Veterinaires. Annals of Veterinary Research*, *11*(4), 445–478.

Kemmerer L. (2015). Chapter 11: Dirty Dancing- Caring for Sloth Bears in India. In L. Kemmerer (Ed), *Bear Necessities: Rescue, Rehabilitation, Sanctuary, and Advocacy*. (pp: 116-127). United Kigndom: Brill.

Kox, L.F., Rhienthong, D., Miranda, A.M., Udomsantisuk, N., Ellis, K., van Leeuwen, J., van Heusden, S., Kuijper, S. & Kolk, A.H. (1994). A more reliable PCR for detection of *Mycobacterium tuberculosis* in clinical samples. *Journal of Clinical Microbiology*, 32(3), 672-678.

Lécu, A. & Riquelme, L. (2008). Evolutions des outils diagnostiques de la tuberculose des éspèces sauvages. *Bulletin de l'académie vétérinaire* de France, 161 (2), 151-15.

Lécu, A., & Ball, R. (2011). Mycobacterial infections in zoo animals: Relevance, diagnosis and management. *International Zoo Yearbook*, 45(1), 183–202.

Lécu, A. & Ball, R.L. (2014). Chapter 74- Recent Updates for Ante-mortem Tuberculosis Diagnostics in Zoo Animals. In M.E. Fowler, & E.R. Miller (Eds), *Zoo and Wildlife Medicine, Current therapy, Volume 8*. (pp:703 -508). London, UK: Elsevier Health Sciences.

Lin, P.L., Pawar, S., Myers, A., Pegu, A., Fuhrman, C., Reinhart, T.A., Capuano, S.V., Klein, E. & Flynn, J.L. (2006). Early Events in *Mycobacterium tuberculosis* Infection in Cynomolgus Macaques. *Infection and Immunity* 74 (7), 3790–3803.

Lin, P.L., Plessner, H.L., Voitenok, N.N. & Flynn, J.L. (2007). Tumor Necrosis Factor and Tuberculosis. *Journal of Investigative Dermatology Symposium Proceedings*, 2007 (12), 22–25.

Lyashchenko, K., Colangeli R., Houde M., Jahdali, H.A., Menzies D. & Gennaro, M.L. (1998). Heterogenous antibody responses in tuberculosis. *Infection and Immunity*, 66, 3936–3940.

Lyashchenko, K.P., Singh, M., Colangeli, R, Gennaro, M.L. (2000). A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. *Journal of Immunological Methods*, 242(1-2), 91-100.

Lyashchenko,K., Greenwald,R., Esfandiari,J., Olsen,J.H., Ball,R., Dumonceaux,G., Dunker,F., Buckley,C., Richard,M., Murray,S., Payeur,J., Andersen,P., Pollock,J.M., Mikota,S., Miller,M., Sofranko,D. & Waters, W.R. (2006).Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. *Clinical and Vaccine Immunology*, 13, 722-732.

Lyashchenko K. P., Greenwald, R., Esfadiari, J., Greenwald, D., Nacy, C. A., Gibson, S., Didier, P. J., Washington, M., Szczerba, P.& Motzel, S. (2007). PrimaTB STAT-PAK assay, a novel, rapid lateral-flow test for tuberculosis in nonhuman primates. *Clinical and Vaccine Immunology*, 14, 1158–1164.

Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Chamber, M.A., Vicente, J., Gortazar, C., Santos, N., Correia-Neves, M., Buddle, B.M., Jackson, R., O'Brien, D.J., Schmitt, S., Palmer, M.V., Delahay, R.J. & Waters, W.R. (2008). Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife. *Veterinary Microbiology,* 132(3–4): 283–292.

Maas, M., Michel, A.L., & Rutten, V.P. M.G. (2013). Facts and dilemmas in diagnosis of tuberculosis in wildlife. *Comparative Immunology, Microbiology and Infectious Diseases*, 36(3), 269–285.

Martín-Atance, P., León-Vizcaíno, L., Palomares, F., Revilla, E., González-Candela, M., Calzada, J., Cubero-Pablo, M. J. & Delibes, M. (2006). Antibodies to Mycobacterium bovis in wild carnivores from Doñana National Park (Spain). *Journal of Wildlife Diseases*, 42(3), 704–708.

Martin, L.B. (2009). Stress and immunity in wild vertebrates: Timing is everything. *General and Comparative Endocrinology*, 163(1-2), 70–76.

Martinho, A.P.V., Franco, M.M.J., Ribeiro, M.G., Perrotti, I.B.M., Mangia, S.H., Megid, J., Vulcano, L.C., Lara, G.H.B., Santos, A.C.B., Leite, C.Q.F., Sanches, O.C. & Pae, A.C. (2013). Case Report: Disseminated *Mycobacterium tuberculosis* Infection in a Dog. *American Journal of Tropical Medicine and Hygine*, 88(3), 596-600.

Maslow, J.N. & Mikota, S.K. (2015). Tuberculosis in Elephants- A Reemergent Disease: Diagnostic Dilemmas, the Natural History of Infection, and New Immunological Tools. *Veterinary Pathology*, 52(3), 437-440.

Mazurek, G.H., Jereb, J., Vernon, A., LoBue, P., Goldberg, S. & Castro, K. (2011). TB Elimination- Interferon-Gamma Release Assays (IGRAs) – Blood Tests for TB Infection. *Division of Tuberculosis Elimination, National Center for HIV, STD, and TB Prevention, CDC.* Accessed on December 20th, 2016, available at https://www.cdc.gov/tb/publications/factsheets/testing/IGRA.pdf.

McGraw-Hill Dictionary of Scientific & Technical Terms (6th ed.). (2003). NY, USA: McGraw-Hill Professional Publishing Accessed November 24th, 2016, available at http://encyclopedia2.thefreedictionary.com/Canoidea.

Mehrotra, P.K., Bhargava, S., Choudhary, S., Mathur, B.B.L. (1999). Tuberculosis in sloth bear at Jaipur Zoo. *Zoos' Print Journal* 14(7), 68-68.

Michalak, K., Austin, C., Diesel, S., Bacon, J.M., Zimmerman, P., Maslow, J.N. (1998). *Mycobacterium tuberculosis* infection as a Zoonotic Disease: Transmission between Humans and Elephants*. Emerging Infectious Dieases*, 4(2), 283-287.

Michel, A. L., Venter, L., Espie, I. W., & Coetzee, M. L. (2003). Mycobacterium tuberculosis infections in eight species at the National Zoological Gardens of South Africa, 1991-2001. *Journal of Zoo and Wildlife Medicine : Official Publication of the American Association of Zoo Veterinarians*, 34(4), 364–370.

Michel, A.L., Muller, B. & van Helden, P.D. (2010). *Mycobacterium bovis* at the animal-human interface: a problem, or not? *Veterinary Microbiology*, 140 (3–4), 371–381.

Mikota, S.K., Larsen, R.S. & Montali, R.J. (2000). Tuberculosis in Elephants in North America. *Zoo Biology*, 19, 393-403.

Mikota, S.K., (2008). Chapter 43: Tuberculosis in Elephants. In M.E. Fowler, & E.R. Miller (Eds), *Zoo and Wildlife Medicine, Current therapy, Volume 6*. (pp 355-364). London, UK: Elsevier Health Sciences.

Miller, M. (2008). Chapter 2: Current Diagnostic Methods for Tuberculosis in Zoo Animals. In M.E. Fowler, & E.R. Miller (Eds), *Zoo and Wildlife Medicine, Current therapy, Volume 6*. (pp 10-19). London, UK: Elsevier Health Sciences.

Montali, R.J., Mikota, S.K. & Cheng, L.I. (2001). *Mycobacterium tuberculosis* in zoo and wildlife species. *Scientific and Technical Review of the Office International des Epizooties*, 20(1), 291-303.

National Institute for Health and Care Excellence (NICE). (2016). Tuberculosis: Prevention, diagnosis, management and service organisation. *TB Clinical Guideline Update*. Accessed on August 10th, 2016, available at http://www.ncbi.nlm.nih.gov/books/NBK338750/pdf/Bookshelf_NBK338750.pdf.

Nolte, F.S. & Metchock, B. (1995). *Mycobacterium*. In P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, R.H. Yolken (Eds), *Manual of Clinical Microbiology*. (6th ed). (pp. 400–437). Washington, DC: American Society for Microbiology.

Olsen, I., Barletta, R.G. & Thoen, C.O. (2010). Chapter 7: *Mycobacterium*. In C. L. Gyles, J. F. Prescott, J. G. Songer & C.O. Thoen (Eds), *Pathogenesis of Bacterial Infections in Animals*. (4th ed.). (pp: 113-132). Oxford, UK: Wiley-Blackwell.

Ong, C. W.M., Elkington, P.T., & Friedland, J.S. (2014). Tuberculosis, Pulmonary Cavitation, and Matrix Metalloproteinases. *American Journal of Respiratory and Critical Care Medicine*, 190(1), 9–18.

Padmini Salgame, P. (2011). MMPs in tuberculosis: granuloma creators and tissue destroyers. *The Journal of Clinical Investigation*, 121(5), 1686-1688.

Parsons, S.D.C, Warren, R.M., Ottenhoff, T.H.M., van Pittius, N.C. & van Helden, P.D. (2012). Detection of *Mycobacterium tuberculosis* infection in dogs in a high-risk setting. *Research in Veterinary Science,* 92(3), 414–419.

Paul, M. (2014). Pet Health Network: Tuberculosis in Dogs and Cats. Acessed on November 14th, 2016, available at http://www.pethealthnetwork.com/dog-health/dog-diseases-conditions-a-z/tuberculosis-dogs-and-cats.

Pfyffer, G. E. (2007). *Mycobacterium*: General Characteristics, Laboratory Detection, and Staining Procedures. In P. R. Murray (Ed.). *Manual of Clinical Microbiology* (9th ed.). (pp. 543-572). Washington DC: ASM Press.

Phillips, T. (2016). Biotech and Pharmaceutical Industries. RFLP: Definition and DNA Analysis Applications. Accessed on November 14th, 2016, available at https://www.thebalance.com/rflp-definition-and-dna-analysis-applications-375574.

Phunpae, P., Chanwong, S., Tayapiwatana, C., Apiratmateekul, N., Makeudom, A. & Kasinrerk, W. (2014). Rapid diagnosis of tuberculosis by identification of Antigen 85 in mycobacterial culture system. *Diagnostic Microbiology and Infectious Disease*, 78, 242–248.

Pocock, R. I. (1933). The black and brown bears of Europe and Asia: Part II. *Journal of Bombay Natural History Society*, 36: 101-138

Rastogi, N., Legrand, E. & Sola, C. (2001). The mycobacteria: an introduction to nomenclature and pathogenesis. *Scientific and Technical Review of the Office International des Epizooties*, 20 (1), 21-54.

Renukaprasad, C., Arun, A.S., Kiran, C.M., Rahul S. & Selvaraj, I. (2013). Sampling technique for disease diagnosis with reference to *Mycobacterium tuberculosis* in a large population of rescued dancing Sloth bears. In G.S. Parida, N. Panda, B. Mohanty, D. Das, K. Behura, M. Dehuri (Eds), *Proceedings of the Souvenir National Symposium: "Newer approaches in welfare and health management of captive and free ranging wild animals", Bhubaneswar, India, 19-21 April, pp: n.a.*

Riquelme, L. (2009). La tuberculose chez les espèces sauvages captives: possibilité d'utilisation du test interferon gamma pour son diagnostic *ante-mortem*. *Thèse Med. Vet*. *Alfort,* 35, 239-239.

Rishikesava, R., Arun, A.S., Chandranaik, B.M., Basavarajappa, P., Giridhar P. & Renukaprasad, C. (2008). Study on Prevalence of Tuberculosis in Rescued Captive Sloth Bears (*Melursus ursinus*). *Journal of Veterinary Public Health*, 6(1), 53-54.

Sakamoto, K. (2012). The Pathology of *Mycobacterium tuberculosis* Infection. *Veterinary Pathology,* 49(3), 423-439.

Seshamani, G. & Satyanaraya K. (1997). *The Dancing Bears of India*. Accessed April 6th, 2016, available for download at http://wildlifesos.org/publications/.

Schluger, N.W. & Rom, W.N. (1998). The Host Immune Response to Tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, 157, 679-691.

Schneider, G., Kack, H. & Lindquist, Y. (2000). The manifold of vitamin B6 dependent enzymes. *Structure*, 8, R1–R6.

Snider D. E. (1980). Pyridoxine supplementation during isoniazid therapy. *Tubercle*, 61(4), 191–196.

Somoskovi, A., Gutierrez, C. M. & Salfinger, M. (2008). Laboratory diagnosis of tuberculosis: novel and nonconventional methods. *Reviews in Medical Microbiology*, 19, 19–38.

Stein, C. M. (2012). Genetics of Susceptibility to Tuberculosis. *Encyclopedia of Life Sciences (eLS) John Wiley & Sons*. Accessed on May 3rd, 2016, available at http://www.els.net/WileyCDA/ElsArticle/refId-a0023886.html

Thoen, C.O. (1993). Chapter 11: TB and other mycobacterial disease in captive wild animals. In M.E. Fowler, & E.R. Miller (Eds), *Zoo and Wildlife Medicine, Current therapy, Volume 3*. (pp 45-49). London, UK: Elsevier Health Sciences.

van der Kuyp, F., Mahan, C.S. (2012). Prolonged positivity of sputum smears with negative cultures during treatment for pulmonary tuberculosis. *International Journal of Tuberculosis and Lung Disease*, (12),1663–1667.

Vargas, D., García. L., Gilman R.H., Evans C., Ticona E., Ñavincopa M., Luo R.F., Caviedes, L., Hong, C. & Moore, D.A.J. (2005). Diagnosis of sputum-scarce HIV-associated pulmonary tuberculosis in Lima, Peru. *Lancet*, 365, 150–152.

Von Hohendorff, R. & Giacomini, C. (2014). Chapter 41: Carnivora- Ursidae. In Z.S. Cubas, J.C.R. Silva & J.L. Catão-Dias (Eds), *Tratado de Animais Selvagens- Medicina Veterinária, Volume 1.* (2nd ed). (pp: 880-892). São Paulo, Brasil: Editora Roca.

United States Animals Health Association: Elephant Tuberculosis Subcommittee. (2012). Guidelines for the Control of Tuberculosis in Elephants 2012. Accessed on May 22nd, 2016, available at http://www.usaha.org/Portals/6/Committees/tuberculosis/TB%20Guidelines%202012%20Draft%20revision%2020April2012.pdf.

Verma-Kumar, S., Abraham, D., Dendukuri, N., Cheeran, J. V., Sukumar, R., & Balaji, K. N. (2012). Serodiagnosis of Tuberculosis in Asian Elephants (*Elephas maximus*) in Southern India: A Latent Class Analysis. *Plos One*, 7 (11).

Vervenne, R.A.W., Jones, S.L., Van Soolingen, D., Van Der Lann, T., Andersen P., Heidt, P.J., Thomas, A.W. & Langermans, J.A.M. (2004). TB diagnosis in non-human primates: comparison of two interferon-g assays and the skin test for identification of *Mycobacterium tuberculosis* infection. *Veterinary Immunology and Immunopathology*, 100, (61–71).

World Health Organization. (2015). *2015 Global Tuberculosis Report.* Accessed on June 28th, 2016, available at http://apps.who.int/iris/bitstream/10665/191102/1/9789241565059_eng.pdf?ua=1.

World Health Organization. (2016). *2016 Global Tuberculosis Report.* Accessed on October 20th, 2016, available at http://apps.who.int/iris/bitstream/10665/250441/1/9789241565394-eng.pdf?ua=1 .

Yoganadn, K., Rice, C.G. & Johnsinhg, A.J.T. (2013). Chapter 25: Sloth bear (*Melursus ursinus*). In A.J.T Johnsinhg & N. Manjrekar (Eds), *Mammals of South Asia*. (pp: 438- 4). India: Universities Press.